US009226645B2

(12) **United States Patent**
Ntziachristos

(10) **Patent No.: US 9,226,645 B2**
(45) **Date of Patent: Jan. 5, 2016**

(54) **SYSTEM AND METHOD FOR NORMALIZED DIFFUSE EMISSION EPI-ILLUMINATION IMAGING AND NORMALIZED DIFFUSE EMISSION TRANSILLUMINATION IMAGING**

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(73) Assignee: **The General Hospital Corporation**, Boston, MA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 1327 days.

(21) Appl. No.: **12/973,075**

(22) Filed: **Dec. 20, 2010**

(65) **Prior Publication Data**

US 2011/0087111 A1 Apr. 14, 2011

Related U.S. Application Data

(62) Division of application No. 11/720,967, filed as application No. PCT/US2005/044651 on Dec. 8, 2005, now abandoned.

(60) Provisional application No. 60/634,369, filed on Dec. 8, 2004.

(51) **Int. Cl.**
A61B 6/00 (2006.01)
A61B 1/00 (2006.01)
(Continued)

(52) **U.S. Cl.**
CPC ***A61B 1/00009*** (2013.01); ***A61B 5/0064*** (2013.01); ***A61B 5/0071*** (2013.01); ***A61B 5/0084*** (2013.01); ***A61B 5/0088*** (2013.01); ***A61B 5/415*** (2013.01); ***A61B 5/418*** (2013.01); *A61B 1/043* (2013.01)

(58) **Field of Classification Search**
CPC A61B 17/00; A61B 17/02; A61B 17/16
USPC 600/476, 478
See application file for complete search history.

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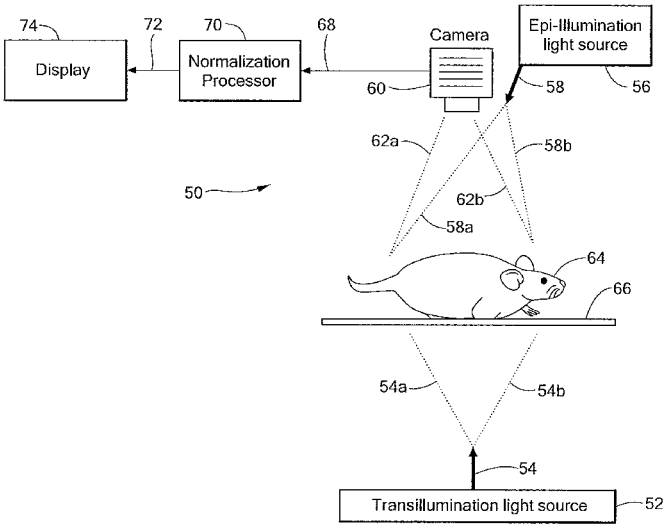
(57) **ABSTRACT**

A system and method provide normalized fluorescence epi-illumination images and normalized fluorescence transillumination images. The normalization can be used to improve two-dimensional (planar) fluorescence epi-illumination images and two-dimensional (planar) fluorescence transillumination images. The system and method can also provide normalized bioluminescence epi-illumination images and normalized bioluminescence transillumination images. In some arrangements, the system and method can provide imaging of small animals, intro-operative imaging, endoscopic imaging, and/or imaging of hollow organs.

28 Claims, 13 Drawing Sheets



(51) **Int. Cl.**

A61B 5/00	(2006.01)
A61B 1/04	(2006.01)

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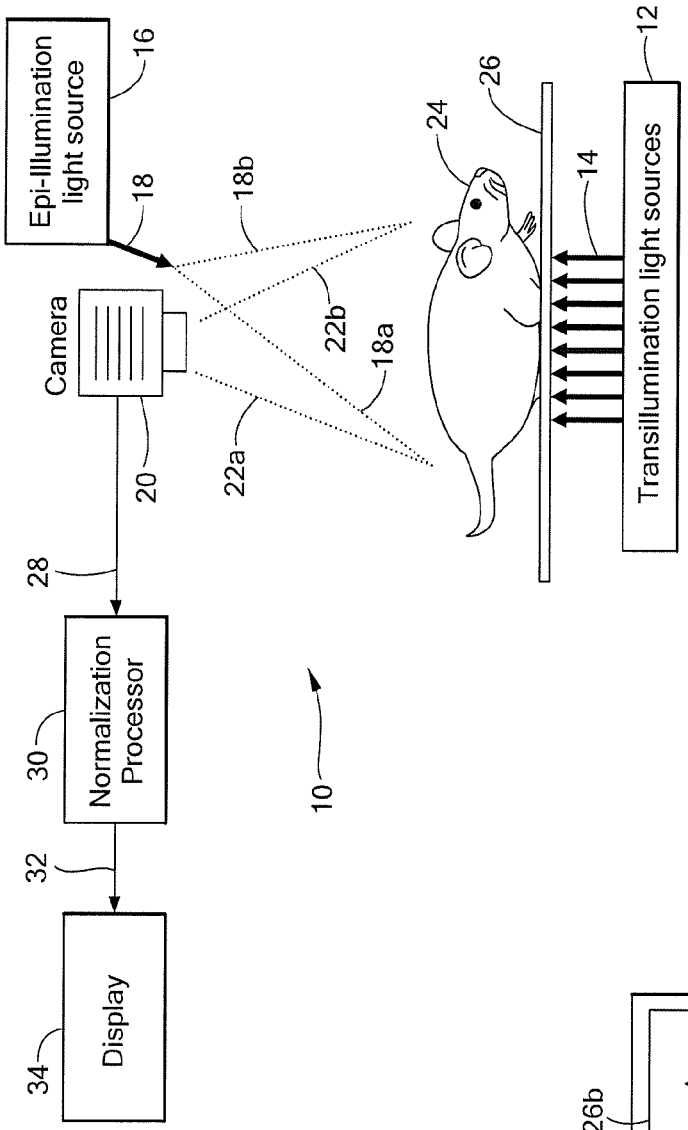
Epi-Illumination light source
16
18
18b
22b
18a
22a
Camera
20
28
Normalization Processor
30
32
Display
34
10
24
26
14
12
Transillumination light sources

FIG. 1

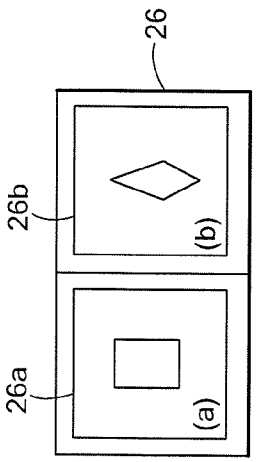
26
26b
(b)
26a
(a)

FIG. 1A

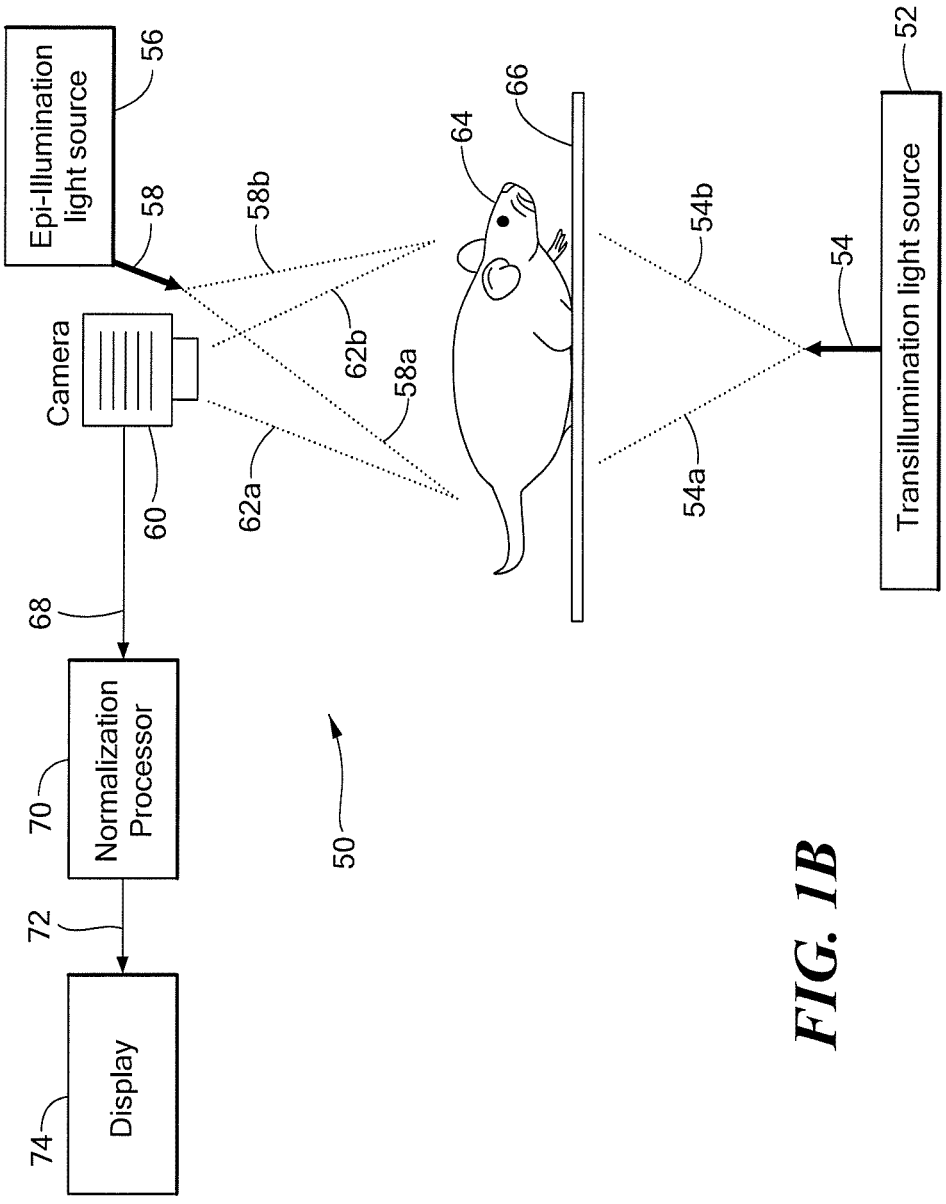
Epi-Illumination light source
56
58
58b
62b
58a
62a
Camera
60
68
Normalization Processor
70
72
Display
74
50
64
66
54b
54a
54
Transillumination light source
52

FIG. 1B

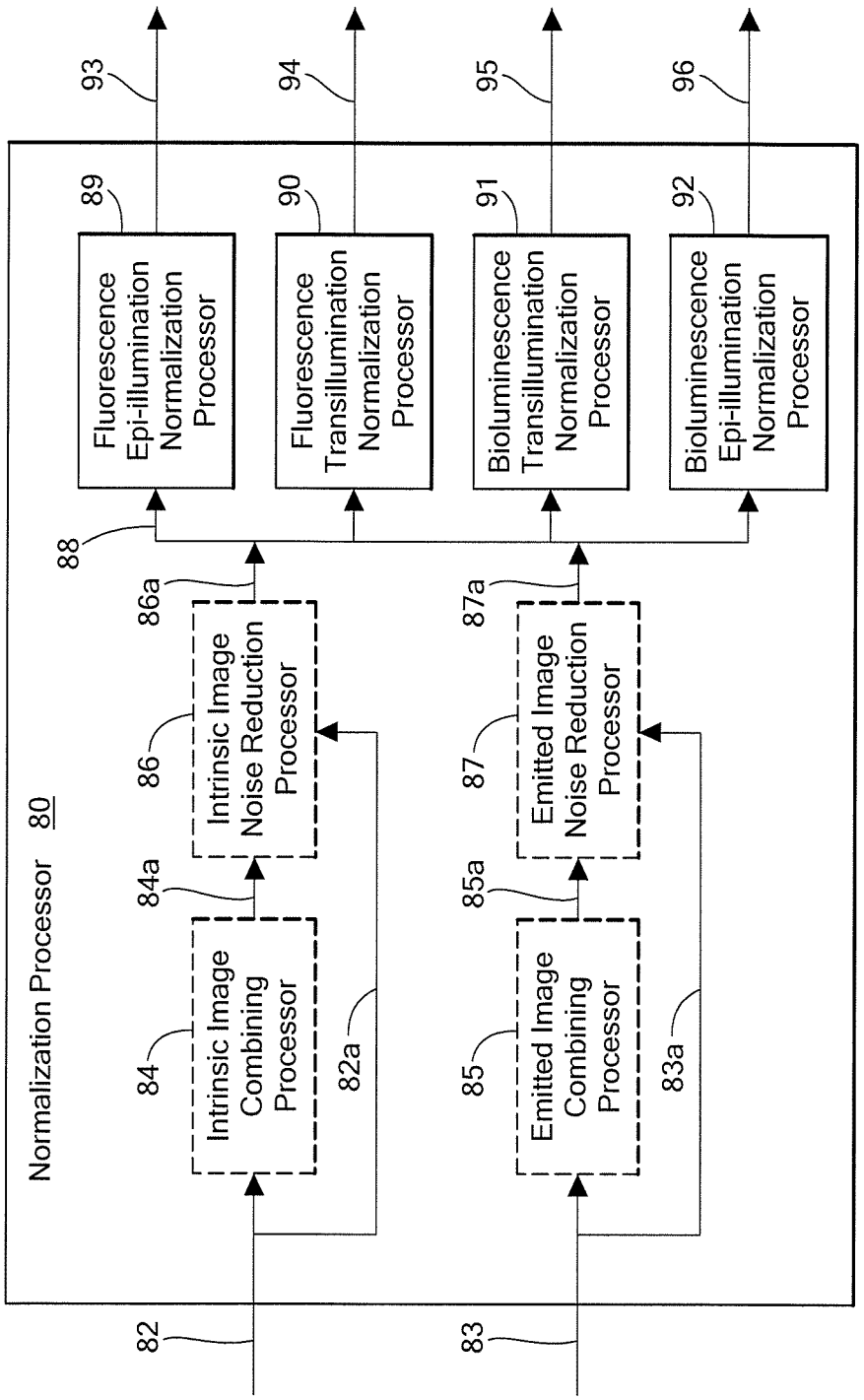
Normalization Processor 80
82
83
84
Intrinsic Image Combining Processor
84a
85
Emitted Image Combining Processor
85a
86
Intrinsic Image Noise Reduction Processor
86a
87
Emitted Image Noise Reduction Processor
87a
88
89
Fluorescence Epi-illumination Normalization Processor
90
Fluorescence Transillumination Normalization Processor
91
Bioluminescence Transillumination Normalization Processor
92
Bioluminescence Epi-illumination Normalization Processor
93
94
95
96
82a
83a

FIG. 1C

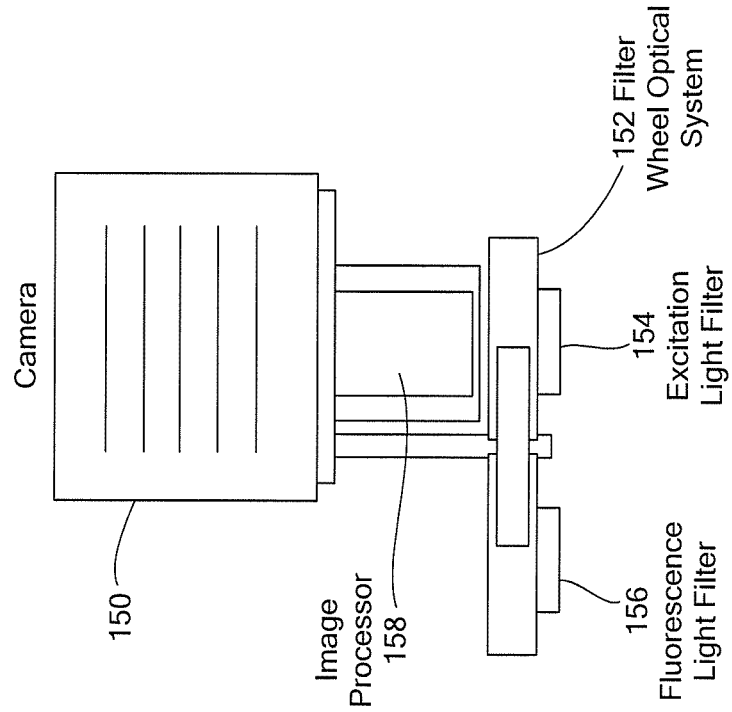
Camera
150
Image Processor 158
152 Filter Wheel Optical System
154 Excitation Light Filter
156 Fluorescence Light Filter

FIG. 2A

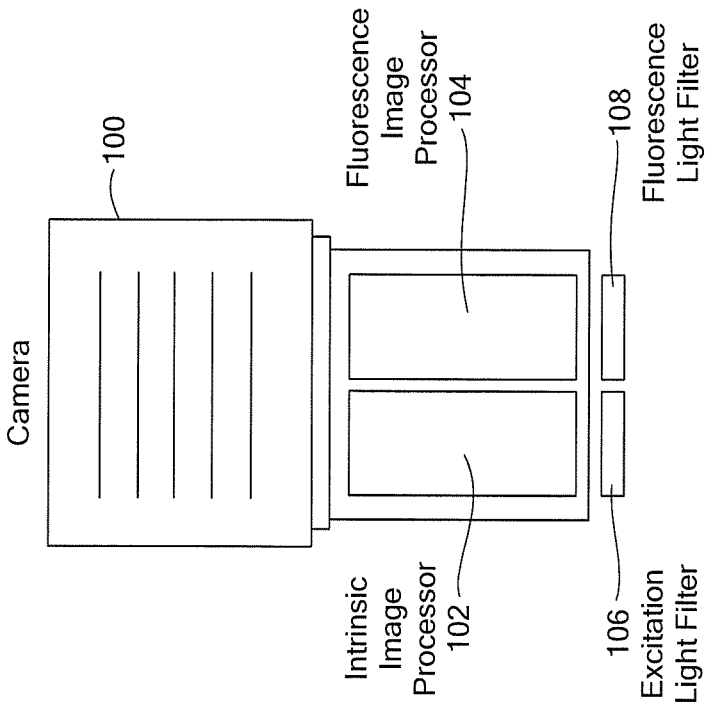
Camera
100
Intrinsic Image Processor 102
Fluorescence Image Processor 104
106 Excitation Light Filter
108 Fluorescence Light Filter

FIG. 2

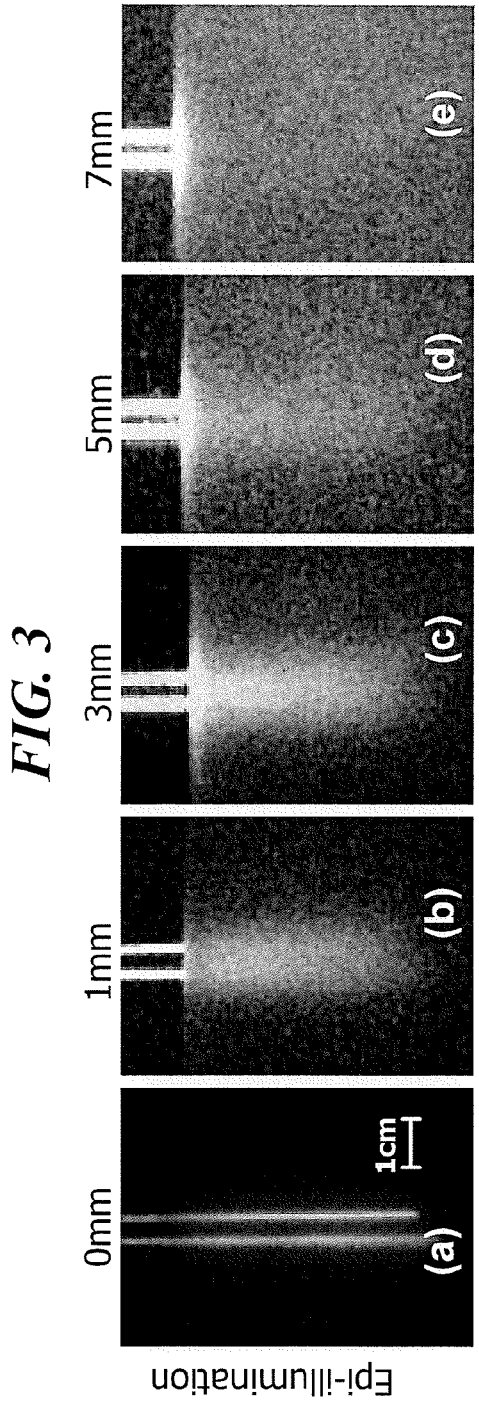
Epi-illumination
0mm
1mm
3mm
5mm
7mm
1cm
(a)
(b)
(c)
(d)
(e)

FIG. 3

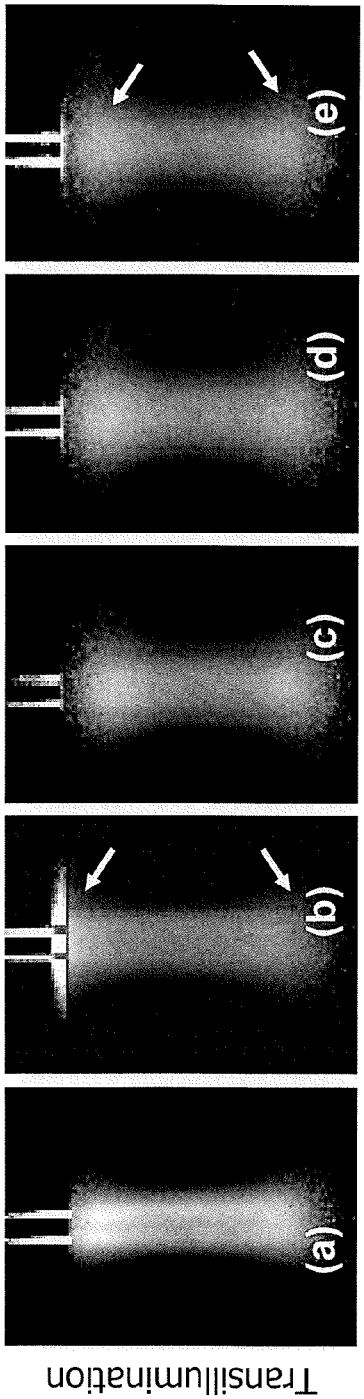
Transillumination
(a)
(b)
(c)
(d)
(e)

FIG. 3A

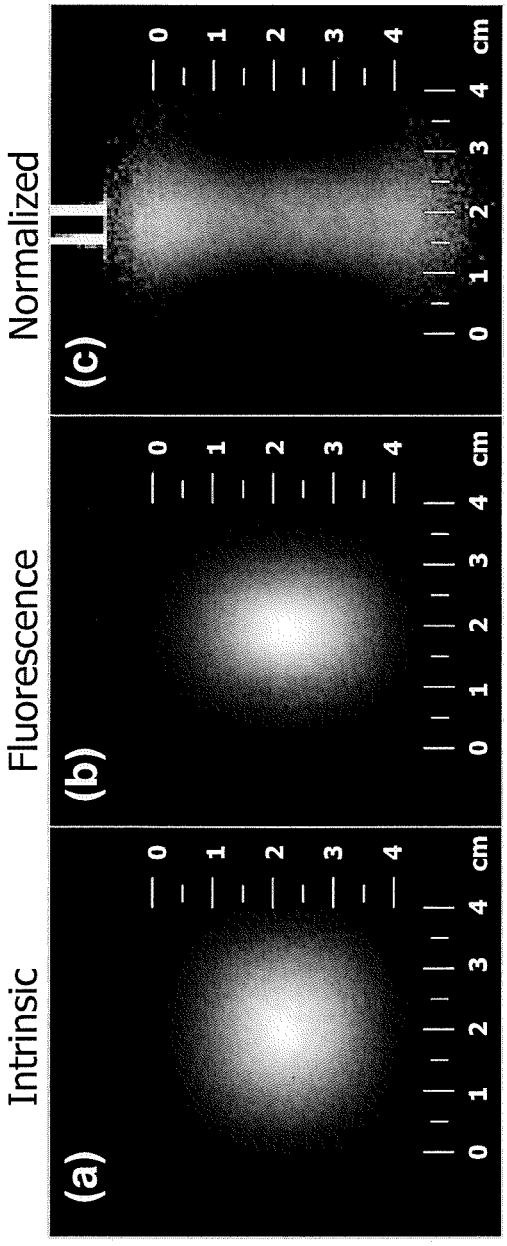

FIG. 4

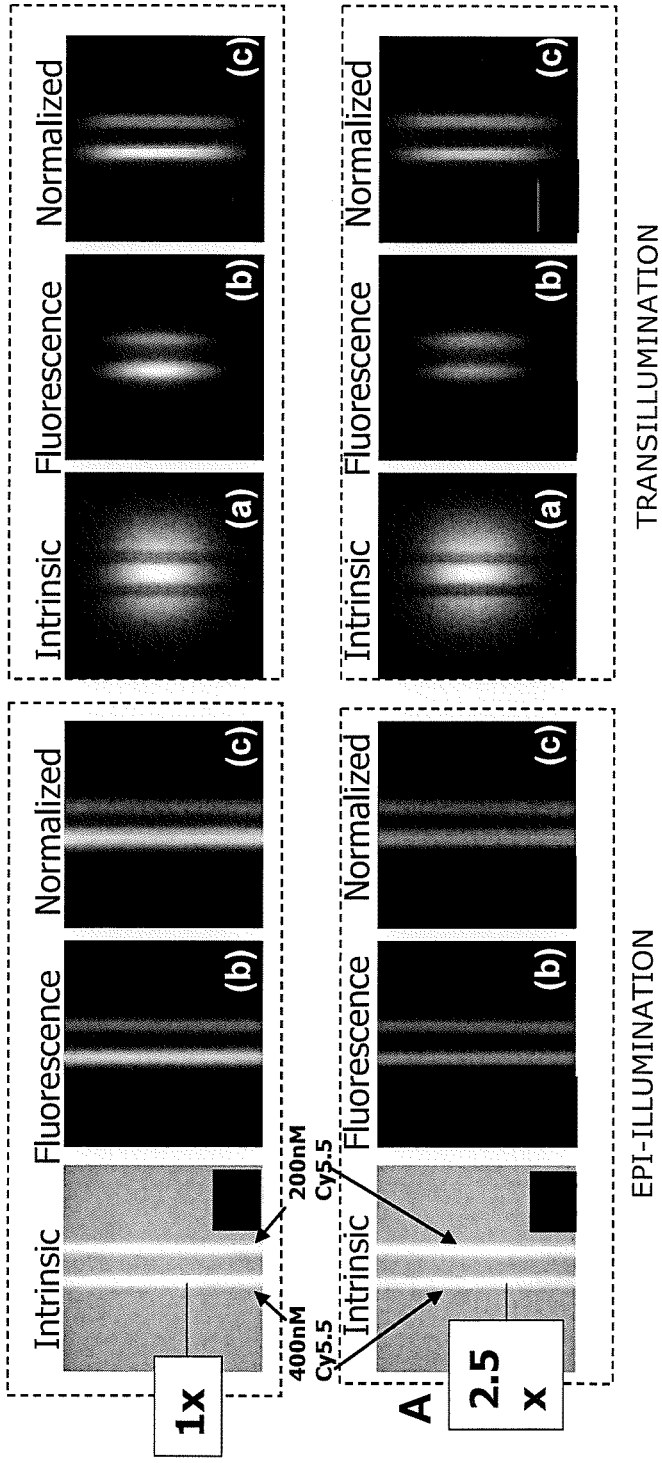

FIG. 5

FIG. 5A

FIG. 5B

FIG. 5C

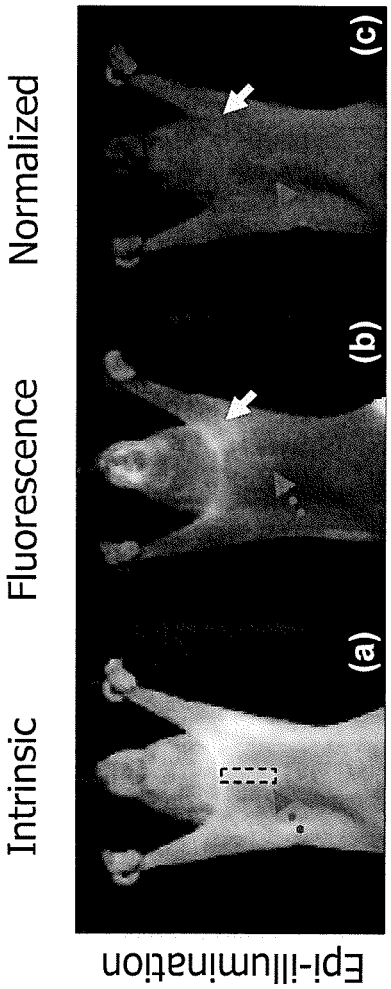
Intrinsic
Fluorescence
Normalized
(a)
(b)
(c)
Epi-illumination

FIG. 6

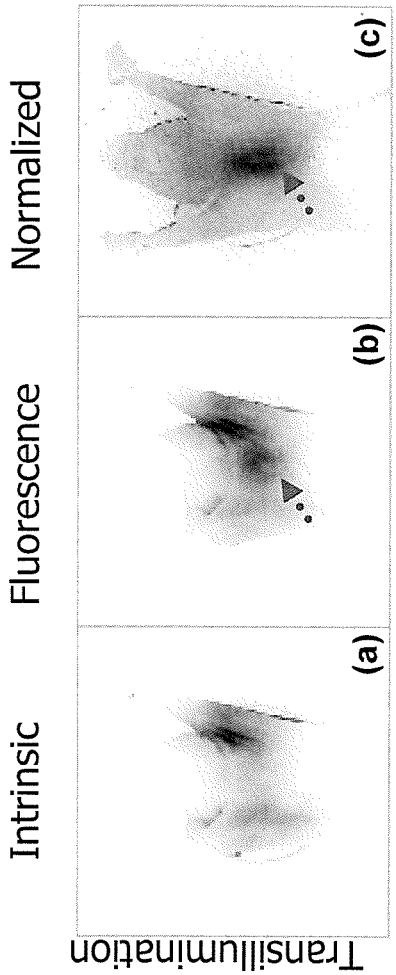
Intrinsic
Fluorescence
Normalized
(a)
(b)
(c)
Transillumination

FIG. 6A

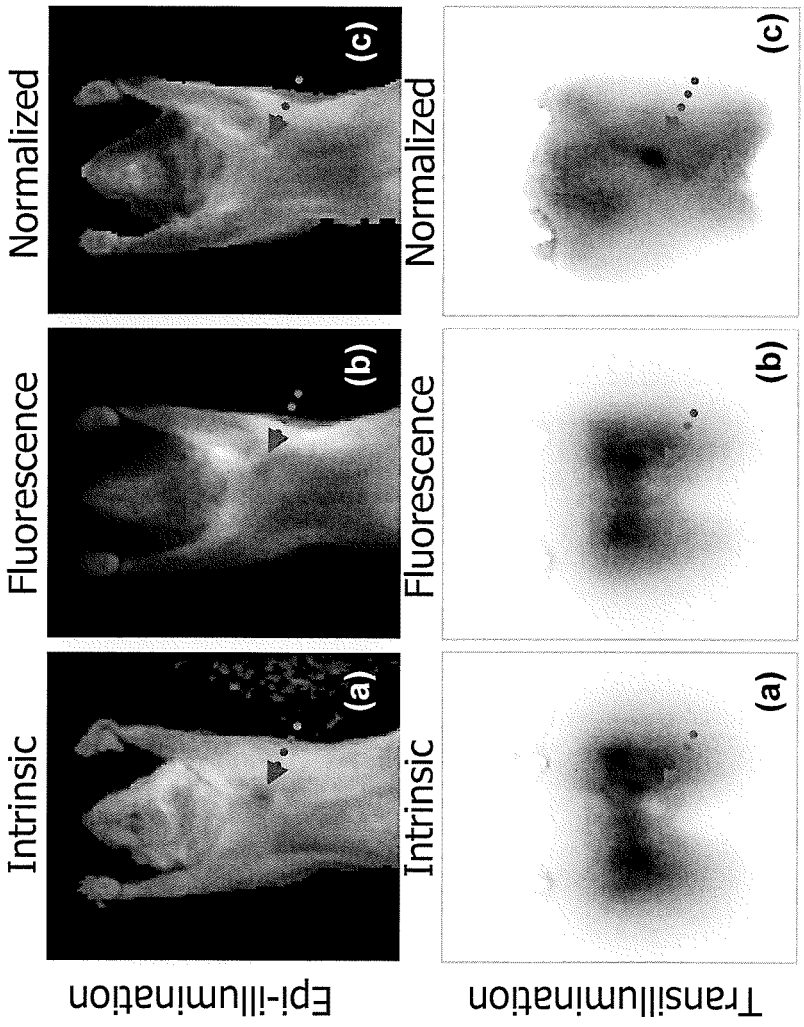
Epi-illumination
Transillumination
Intrinsic
Fluorescence
Normalized
(a)
(b)
(c)

FIG. 7

FIG. 7A

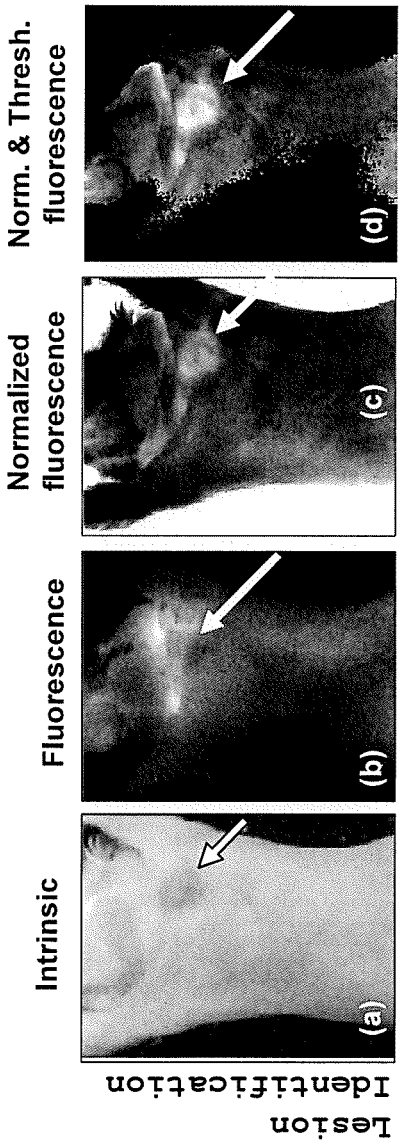
Lesion Identification
Intrinsic
Fluorescence
Normalized fluorescence
Norm. & Thresh. fluorescence
(a)
(b)
(c)
(d)

FIG. 8

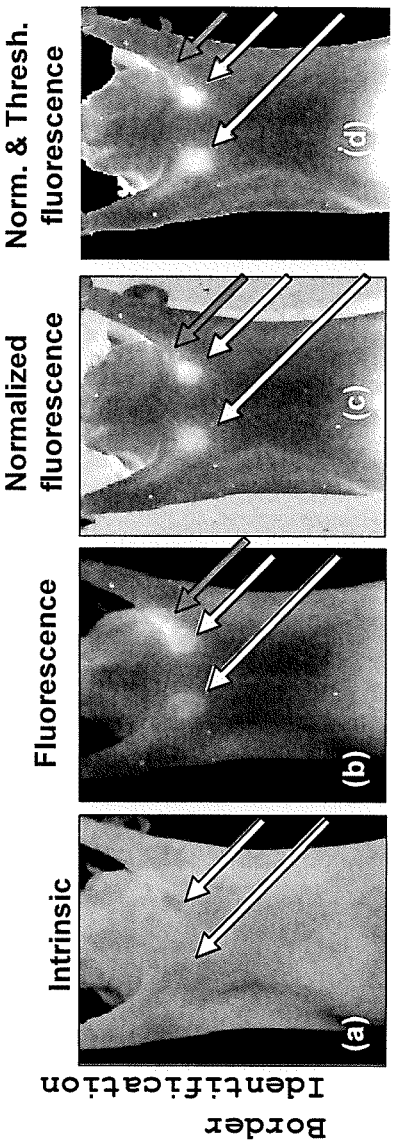
Border Identification
Intrinsic
Fluorescence
Normalized fluorescence
Norm. & Thresh. fluorescence
(a)
(b)
(c)
(d)

FIG. 8A

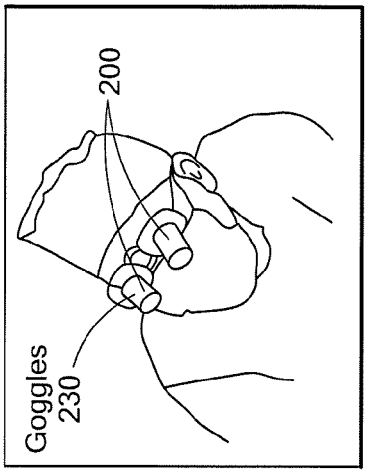
200
Goggles
230

FIG. 9A

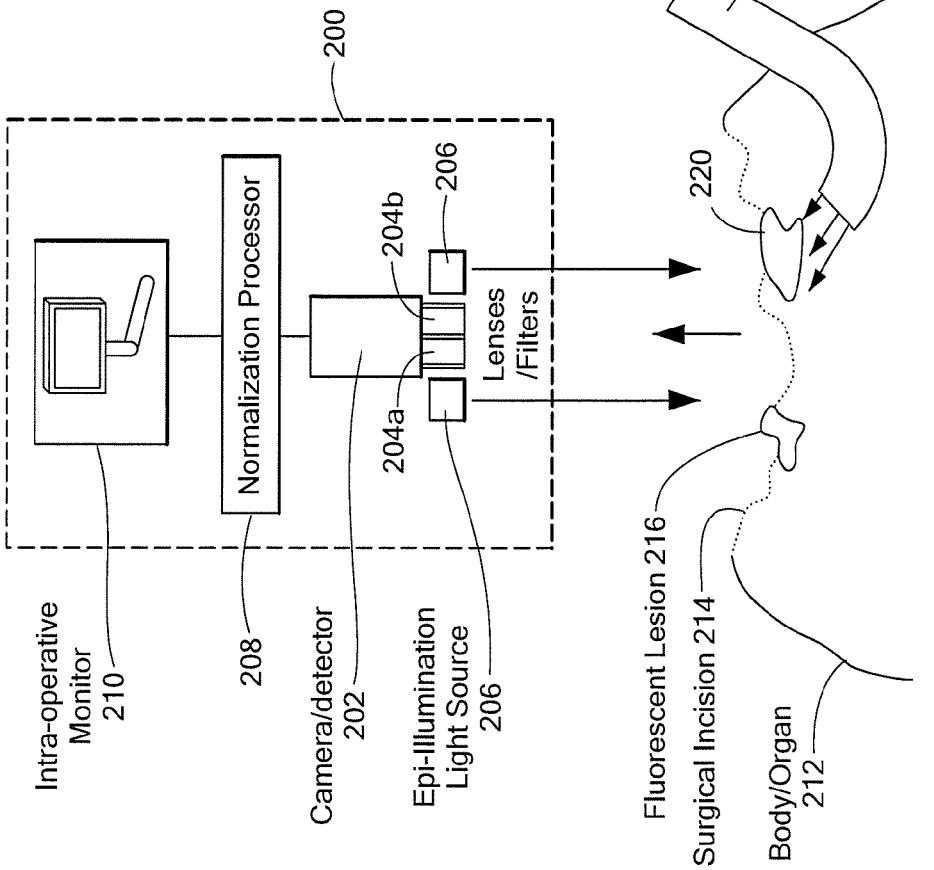
200
Intra-operative
Monitor
210
Normalization Processor
208
Camera/detector
202
204b
204a
206
Epi-Illumination
Light Source
206
Lenses
/Filters
218
Illuminator
Laparoscope/
Endoscope
220
Fluorescent Lesion 216
Surgical Incision 214
Body/Organ
212

FIG. 9

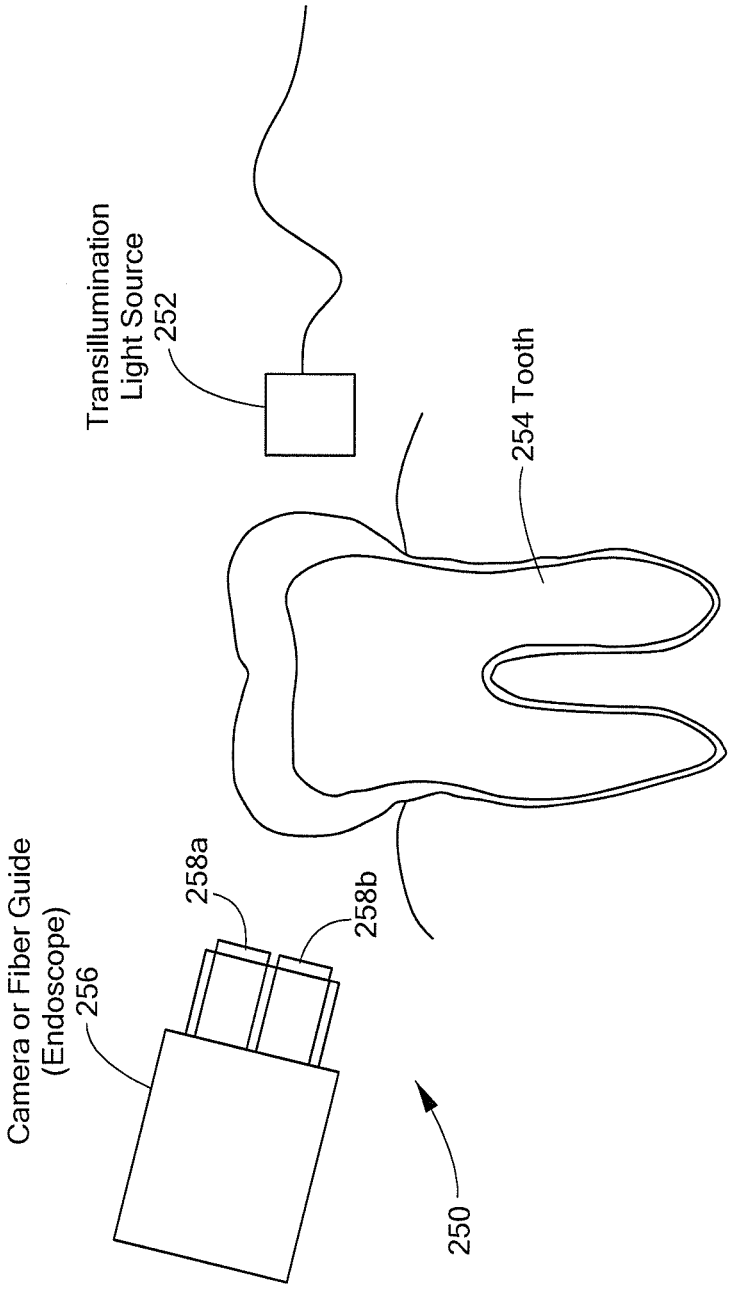
Transillumination
Light Source
252
254 Tooth
Camera or Fiber Guide
(Endoscope)
256
258a
258b
250

FIG. 10

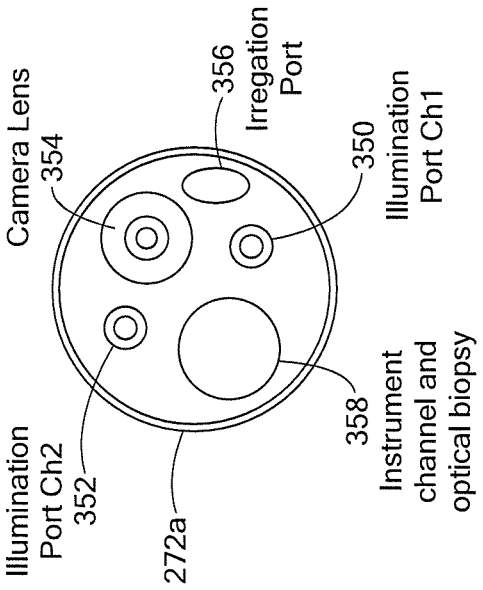

FIG. 11A

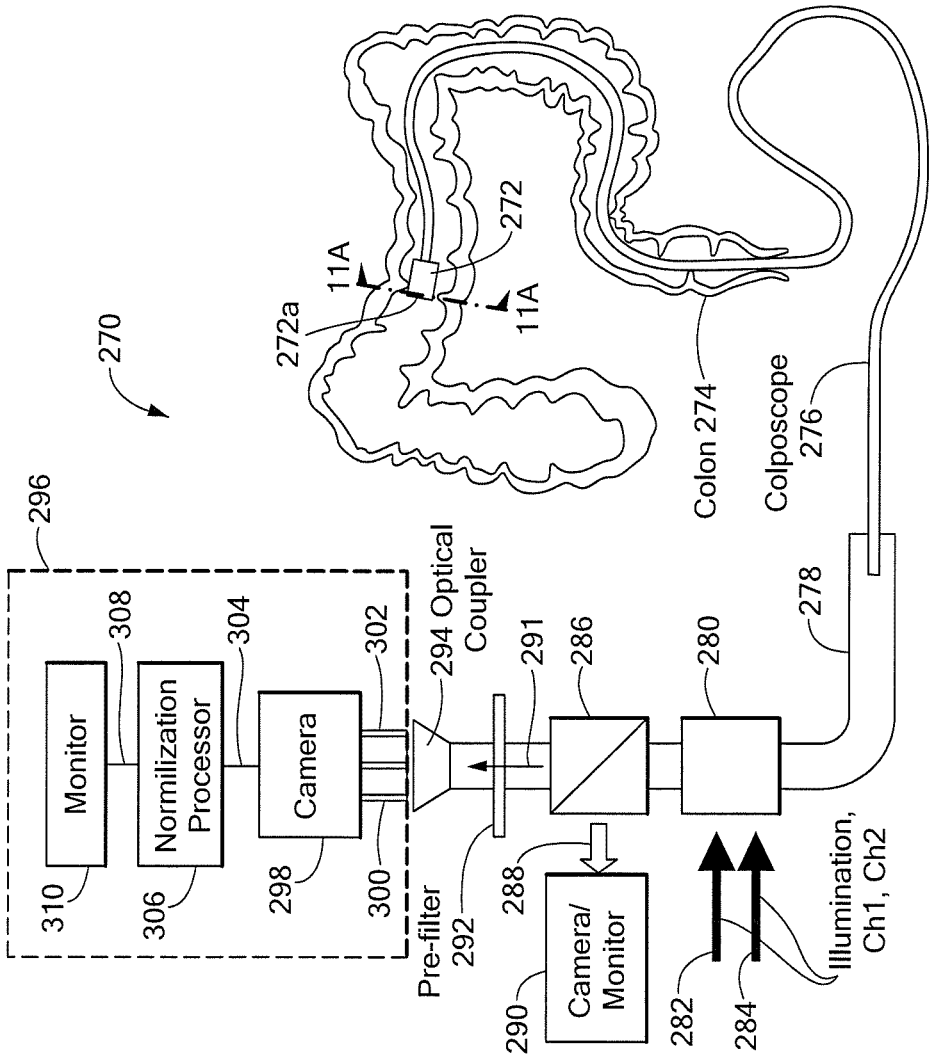

FIG. 11

SYSTEM AND METHOD FOR NORMALIZED DIFFUSE EMISSION EPI-ILLUMINATION IMAGING AND NORMALIZED DIFFUSE EMISSION TRANSILLUMINATION IMAGING

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a Divisional Application of and claims the benefit of U.S. patent application Ser. No. 11/720,967 filed on Jun. 6, 2007, which is a U.S. National Stage application under 35 U.S.C. §371 of, and claims the benefit of, International Patent Application No. PCT/US2005/044651 filed on Dec. 8, 2005, all of which applications claim priority to U.S. Provisional Patent Application No. 60/634,369 filed on Dec. 8, 2004, all of which applications are hereby incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

This invention relates generally to medical imaging and, more particularly, to a system and method for generating fluorescence epi-illumination images, fluorescence transillumination images, and bioluminescence images associated with small animal imaging, intra-operative imaging, endoscopic imaging and imaging of hollow organs.

BACKGROUND OF THE INVENTION

Fluorescence images can be generated in-vivo for imaging of molecular functions and gene expression in live biological tissues. Fluorescence imaging of small animals has been used in biological research, including research in drug discovery and in investigation of fluorescent reporter technologies. Fluorescence imaging has also been used to study various human tissues, for example, tissues exhibiting epithelial diseases, the human breast, joints, and human teeth.

Conventionally, fluorescent light has been used for high-resolution imaging of histological slices of biological tissue using so-called fluorescence microscopy. Fluorescence microscopy is used to provide relatively high-resolution images. However, tissue sectioning used in conventional fluorescence microscopy is limited to slice thicknesses (i.e., tissue depths) on the order of half a millimeter, and therefore, conventional fluorescence microscopy is not appropriate for imaging through entire organs or through the whole human body.

In order to provide images a bit deeper into tissue, conventional systems and techniques have used light sources and fluorochromes that emit near infrared light. The near infrared light is selected because near infrared light has low absorption and can penetrate several centimeters into biological tissue. Near infrared light is used in a variety of optical imaging systems and techniques.

Fluorescent light can be emitted from a tissue in response to an excitation light source transmitting excitation light into the tissue. The excitation light excites the emission of fluorescent light from fluorochromes within the tissue.

Similarly, bioluminescence imaging has been used to image into tissues. The difference between fluorescence and bioluminescence imaging is that, for bioluminescence imaging, no excitation light source is required to cause emission of bioluminescent light. Emission of bioluminescent light in bioluminescence imaging is caused by a chemi-luminescent reaction within the tissue, resulting from transgenes.

The most common macroscopic technique that is conventionally used for fluorescence imaging is fluorescence reflectance imaging (FRI), which is also referred to herein as fluorescence epi-illumination imaging (FEI).

Epi-illumination light sources and epi-illumination imaging are further described below. In general, an epi-illumination light source generates light that is directed toward and then reflects from a surface of biological tissue and/or that propagates into the biological tissue and reflects from internal structures and/or surfaces of the biological tissue. To form an epi-illumination image, image light is collected generally on the same side of the tissue as the epi-illumination light source.

An FEI system transmits light onto and/or into biological tissue and collects the fluorescence light that is emitted back from the tissue, including light that is emitted back from within the tissue. In fluorescence epi-illumination imaging, excitation light (for example, near-infrared light) from an epi-illumination light source is used to illuminate the tissue. The epi-illumination light source is used to excite fluorochromes within the tissue that, in turn, emit fluorescent light. In some arrangements, the emitted light is visible light. In other arrangements, the emitted light is near infra red light. The emitted light can be visually inspected or it can be captured with a CCD camera or other photon detector positioned generally on the same side of the tissue as the epi-illumination light source. Bioluminescence imaging can be similar to fluorescent epi-illumination imaging, but bioluminescence is generated without an epi-illumination light source.

As described above, conventional fluorescence imaging with near-infrared light provides images having relatively low resolution and only small penetration (2-3 mm) of tissue. Higher resolution is achieved when spectral information is utilized and "umixed."

A second method, which has not yet been utilized for research using small animals, but which has found applications in optical breast imaging, uses a transillumination light source to generate transillumination images. Similar to the above-described epi-illumination light source, a transillumination light source generates light that propagates into the tissue. However, unlike epi-illumination light, the transillumination light propagates entirely through the tissue. In transillumination imaging, image light is collected generally on the opposite side of the tissue from the transillumination light source.

Similar to that described above for fluorescence epi-illumination imaging, in fluorescence transillumination imaging, excitation light (for example, near infra red light) from a transillumination light source is used to illuminate a tissue. The excitation light propagates into the tissue, exciting the emission of fluorescent light from within the tissue. However, in contrast to the above-described fluorescence epi-illumination arrangement, in fluorescence transillumination imaging, a CCD camera or other photon detector is positioned generally on the opposite side of the tissue from the transillumination light source. In some arrangements, the emitted light is near infrared light. Fluorescence transillumination imaging (FTI) has been used to visualize functional characteristics of cardiac muscle and in dental diagnostic practice.

In some transillumination arrangements, the transillumination light source and the light detector lie on a virtual line passing through the tissue. In some arrangements the virtual line is generally perpendicular to the tissue and, in other arrangements, the virtual line is not generally perpendicular to the tissue.

Fluorescence epi-illumination imaging (FEI), fluorescence transillumination imaging (FTI), and bioluminescence imaging (BI) are forms of "planar" imaging, which provide two-dimensional images.

More advanced optical imaging systems and methods have been developed, which utilize tomographic methods. These systems and methods operate by obtaining photonic measurements at different projections (i.e., angles) to the tissue and combining the measurements using a tomographic algorithm. Tomography can provide a more accurate image than the above-described forms of planar imaging. Advantages of tomography include an ability for image quantification, an ability to provide two-dimensional or three-dimensional images, an ability to provide three-dimensional imaging with feature depth measurements, and higher sensitivity and higher resolution as compared to planar imaging. In some applications, tomography has been used in-vivo to measure enzyme regulation and treatment response to drugs. In these applications, tomography provides superior imaging performance to planar imaging. However, tomography is more complex than planar imaging, requiring more advanced instrumentation, requiring multiple illumination points (projections), which can require multiple light sources, and requiring advanced theoretical methods for modeling photon propagation in tissues.

SUMMARY OF THE INVENTION

The system and method for normalized epi-illumination imaging and normalized transillumination imaging provide normalization of images generated by planar fluorescence epi-illumination imaging, by planar fluorescence transillumination imaging, by planar bioluminescence epi-illumination imaging, and by planar bioluminescence transillumination imaging. The normalization results in substantially improved images. As further described below, by using a combination of normalized epi-illumination images and normalized transillumination images, each having particular imaging characteristics, still further improvement can be achieved.

In some particular arrangements, as further described below, the system and method can be used during surgery to identify tumors or other lesions and borders thereof. In other words, the system and method can be used for intra-operative imaging. In some arrangements, the system and method can also be used for fluorescence endoscopy and/or laparoscopy. In some arrangements, the system and method can also be used in oral and dental procedures to provide oral or dental images.

In accordance with the present invention, a method of imaging includes generating incident light including excitation light with an excitation light source and directing the incident light toward a tissue. The method also includes receiving the incident light with a light detector after the incident light has interacted with a tissue. The method further includes receiving emitted light with the light detector, wherein the emitted light is emitted from the tissue. The method further includes generating an intrinsic image of the tissue in response to the incident light. The method further includes generating an un-normalized emitted light image of the tissue. The method further includes combining the un-normalized emitted light image and the intrinsic image to generate a normalized emitted light image of the tissue.

The normalized emitted light image generated by the method is a normalized fluorescence epi-illumination image, a normalized fluorescence transillumination image, a normalized bioluminescence epi-illumination image, and/or a normalized bioluminescence transillumination image.

In accordance with another aspect of the present invention, a system for imaging a tissue includes an excitation light source adapted to generate incident light including excitation light. The system also includes a light receiver adapted to receive the incident light after the incident light has interacted with a tissue, further adapted to receive emitted light, wherein the emitted light is emitted from the tissue, further adapted to generate an intrinsic image of the tissue in response to the incident light, and further adapted to generate an un-normalized emitted light image of the tissue. The system further includes a normalization processor adapted to combine the un-normalized emitted light image and the intrinsic image to generate a normalized emitted light image associated with the tissue.

The normalized emitted light image generated by the system is a normalized fluorescence epi-illumination image, a normalized fluorescence transillumination image, a normalized bioluminescence epi-illumination image, and/or a normalized bioluminescence transillumination image.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing features of the invention, as well as the invention itself may be more fully understood from the following detailed description of the drawings, in which:

FIG. **1** is a pictorial of system used for normalized fluorescence epi-illumination imaging and normalized fluorescence transillumination imaging having a normalization processor;

FIG. **1**A is a pictorial showing two light masks that can be used in conjunction with light sources of FIG. **1**;

FIG. **1**B is a pictorial of another system used for normalized fluorescence epi-illumination imaging and normalized fluorescence transillumination imaging having another normalization processor;

FIG. **1**C is a block diagram showing further details of the normalization processors of FIGS. **1** and **1**B;

FIG. **2** is a pictorial showing greater detail of a light detector that can be used in the normalized fluorescence epi-illumination imaging and normalized fluorescence transillumination imaging systems of FIGS. **1** and **1**B;

FIG. **2**A is a pictorial showing greater detail of another light detector that can be used in the normalized fluorescence epi-illumination imaging and normalized fluorescence transillumination imaging systems of FIGS. **1** and **1**B;

FIG. **3** is a series of images of a phantom, generated by the systems of FIGS. **1** and **1**B, when used for normalized fluorescence epi-illumination imaging;

FIG. **3**A is a series of images of the phantom, also seen in FIG. **3**, provided by the systems of FIGS. **1** and **1**B when used for normalized fluorescence transillumination imaging;

FIG. **4** is a series of images of the phantom, also seen in FIGS. **3** and **3**A, showing an intrinsic transillumination image at an excitation light wavelength, a fluorescence transillumination image, and a normalized fluorescence transillumination image;

FIG. **5** is a series of images of a phantom showing an intrinsic epi-illumination image at the excitation light wavelength, a fluorescence epi-illumination image, and a normalized fluorescence epi-illumination image;

FIG. **5**A is another series of images of another phantom, showing an intrinsic epi-illumination image at the excitation light wavelength, a fluorescence epi-illumination image, and a normalized fluorescence epi-illumination image;

FIG. **5**B is a series of images of the phantom, also seen in FIG. **5**, showing an intrinsic transillumination image at the excitation light wavelength, a fluorescence transillumination image, and a normalized fluorescence transillumination image;

FIG. **5**C is another series of images of the phantom, also seen in FIG. **5**A, showing an intrinsic transillumination image

at the excitation light wavelength, a fluorescence transillumination image, and a normalized fluorescence transillumination image;

FIG. **6** is a series of images of a mouse showing an intrinsic epi-illumination image at the excitation light wavelength, a fluorescence epi-illumination image, and a normalized fluorescence epi-illumination image;

FIG. **6**A is a series of images of the mouse of FIG. **6** showing an intrinsic transillumination image at the excitation light wavelength, a fluorescence transillumination, and a normalized fluorescence transillumination image;

FIG. **7** is a series of images of another mouse showing an intrinsic epi-illumination image at the excitation light wavelength, a fluorescence epi-illumination image, and a normalized fluorescence epi-illumination image;

FIG. **7**A is a series of images of the mouse of FIG. **7** showing an intrinsic transillumination image at the excitation light wavelength, a fluorescence transillumination image, and a normalized fluorescence transillumination image;

FIG. **8** is a series of images showing an intrinsic epi-illumination light image at the excitation wavelength, a fluorescence epi-illumination image, a normalized fluorescence epi-illumination image, and a normalized fluorescence epi-illumination image after the application of noise thresholds, showing detection of tumors;

FIG. **8**A is a series of images showing an intrinsic epi-illumination image at the excitation light wavelength, a fluorescence epi-illumination image, a normalized fluorescence epi-illumination image, and a normalized fluorescence epi-illumination image after the application of noise thresholds, showing detection of tumors;

FIG. **9** is a pictorial of a system used for intra-operative imaging, including a portable camera/illuminator combination, a normalization processor, and a monitor, and, in some embodiments, a flexible probe (illuminator/laparoscope/endoscope);

FIG. **9**A is a pictorial of another system used for intra-operative imaging, including goggles that can be worn by a surgeon;

FIG. **10** is a pictorial of a system that can be used for dental imaging;

FIG. **11** is a pictorial of a system used for colposcopy having an colposcopic probe; and

FIG. **11**A is a pictorial showing greater detail of a face of the colposcopic probe of FIG. **11**.

DETAILED DESCRIPTION OF THE INVENTION

Before describing the imaging system and method, some introductory concepts and terminology are explained. As used herein, the term "phantom" is used to describe a test object being imaged. A phantom is typically an article having diffuse light propagation characteristics similar to living tissue, for example, a piece of appropriately engineered resin block. For another example, a phantom can be a vial, which contains cells having fluorescent proteins therein, i.e. a fluorescent marker or a fluorochrome.

As used herein, the term "excitation light" is used to describe light generated by an "excitation light source" that is incident upon a biological tissue. The excitation light can interact with the tissue, and can be received by a light detector (e.g., a camera), at the same wavelength (excitation wavelength) as that at which it was transmitted by the excitation light source. The excitation light can be monochromatic, or it can cover a broader spectrum, for example, white light. The excitation light can be used to generate a so-called "intrinsic excitation light image" of the tissue (or more simply, and intrinsic image), i.e., an image obtained at the same wavelength as the wavelength of the excitation light. The excitation light can also be used to excite fluorescence within the tissue, and to generate a so-called "fluorescence image" of the tissue, at a selected different wavelength than the wavelength of the excitation light.

As used herein, the term "incident light" is used to more generally describe light that is generated by the excitation light source, wherein the incident light can include not only the excitation light having a wavelength selected to excite fluorescence, but also light having other wavelengths. The incident light can include only the excitation light, or it can also include other wavelengths. The wavelengths of the incident light can be generated simultaneously or at different times. The incident light can be used to generate a so-called "intrinsic incident light image" of the tissue (or more simply, an intrinsic image). The intrinsic incident light image is either an image obtained at the same wavelength as the wavelength of the excitation light (i.e., an intrinsic excitation light image), an image obtained at a different wavelength than the wavelength of the excitation light, or an image obtained by a combination of images obtained at a variety of wavelengths, which may or may not include the wavelength of the excitation light. The combination of images is further described below.

In general, it will be understood that an intrinsic image is an image of natural structures inside the tissue, exclusive of any fluorescence of the tissue or of fluorescence generated by fluorescence markers within the tissue. In contrast, a fluorescence image is an image of only the tissue fluorescence or of fluorescence generated by the fluorescence markers within the tissue. The intrinsic image can be either an intrinsic excitation light image, or, more generally, an intrinsic incident light image.

As used herein, the term "epi-illumination light source" is used to describe an excitation light source that can generate a form of excitation light (also referred to herein as "epi-illumination light") that reflects from a surface of biological tissue and/or that propagates into the biological tissue, in order to form a so-called "epi-illumination image." To form an epi-illumination image, image light is collected generally on the same side of the tissue as the epi-illumination light source. An epi-illumination image can be either an intrinsic epi-illumination image (no fluorescence) or a fluorescence epi-illumination image (only fluorescence).

As used herein, the term "transillumination light source" is used to describe an excitation light source that can generate a form of excitation light (also referred to herein as "transillumination light") that propagates into the tissue, in order to generate a so-called "transillumination image." To form a transillumination image, light is collected generally on the opposite side of the tissue from the transillumination light source. Like an epi-illumination image, a transillumination image can be either an intrinsic transillumination image (no fluorescence) or a fluorescence transillumination image (only fluorescence).

In some arrangements, the epi-illumination light source and/or the transillumination light source(s) can, more generally, generate incident light, which includes the excitation light.

To generate an intrinsic epi-illumination image, the excitation light (epi-illumination light) is received by a camera after being directed back from (e.g., reflected from) an object being imaged. In contrast, to generate an intrinsic transillumination image, the excitation light (transillumination light) is be received by a camera after it passes through the object being imaged.

Similarly, to generate a fluorescence epi-illumination image, the excitation light (epi-illumination light) excites fluorescence on or in the tissue, which is directed back from tissue being imaged, and which is received at a wavelength different from the excitation light. To generate a fluorescence transillumination image, the excitation light (transillumination light) also excites fluorescence in the tissue, which is directed through the tissue being imaged, and which is received at a wavelength different from the excitation light.

As used herein, the term "emitted light" is used to describe light generated by or within a biological tissue. As used herein, the term "fluorescence" is used to describe a form of emitted light generated via excitation of a marker fluorochrome in response to the excitation light. As used herein, the term "bioluminescence" is used to describe another form of emitted light emitted from a tissue, generally in the absence of the excitation light. As used herein, the term "emitted light image" is used to describe either a fluorescence image or a bioluminescence image.

As used herein, the term "image" is used to describe a visual presentation of something such as an object or a scene. An image may be represented as image data, which can be displayed on a computer monitor. Image data can be generated by a digital camera or other image device and provided to a computer system or other processing device. However, it will be understood that the term "image," as used herein, is also used to refer to the image data.

The system and method described herein to explain inventive concepts refer to the use of particular types of light or light having particular characteristics. For example, reference is made to systems and methods using near-infrared excitation light, which provides particular benefits in the near-infrared (NIR) wavelength range of about 650-1000 nm. It should, however, also be appreciate that the system and method described herein can also be applied to excitation light having other wavelengths, for example to light in the visible range of about 400 nm-650 nm. Also, the system and method apply to a system in which excitation light is generated by an excitation light source in one wavelength range, for example, in the visible range, and the fluorescent light emitted by fluorochromes is in another wavelength range, for example in the NIR range. The system and method also apply where both the excitation light generated by the excitation light source and the light emitted by the fluorochromes are in the NIR range. In addition, excitation light and/or emitted light having a wavelength at the interface of visible range and near-infrared range can be used, for example in the 550 nm-650 nm range. Some wavelengths are particularly beneficial for bioluminescence imaging and for imaging of red-shifted fluorochromes and fluorescent proteins. The excitation light can have the same intensity at all wavelengths therein, or it can have predetermined attenuation of selected wavelengths, e.g., by using appropriate filters. Also, excitation light beyond the wavelength range of 400 nm to 1000 nm can be used.

The term "fluorochrome" as used herein will be understood to describe a type of known biocompatible dye or other fluorescence agent that can be systemically or topically applied to a biological tissue. Some fluorochromes are target fluorchromes, which tend to congregate at certain anatomical, functional, or molecular features in the tissue, including for example, at cancerous lesions.

Referring now to FIG. **1**, an imaging system **10** includes a plurality of transillumination light sources **12** adapted to generate excitation (transillumination) light, which is represented by a plurality of arrows **14**. The system **10** also includes an epi-illumination light source **16** adapted to generate excitation (epi-illumination) light represented by an arrow **18** and lines **18***a*, **18***b*, which are generally indicative of light beam boundaries. Thus, the epi-illumination light source **16** emits a single relatively broad beam of light **18**, **18***a*, **18***b*. The transillumination light **14** and the epi-illumination light **18**, **18***a*, **18***b* are directed toward a biological tissue **24**. In this particular example, the biological tissue is shown to be a mouse **24**.

In some embodiments, an optional light mask **26** can be disposed between the transillumination light sources **12** and the biological tissue **24**. Thus, in those embodiments, which utilize a light mask such as light mask **26**, the transillumination light **14** passes through the light mask **26**, and impinges upon the biological tissue **24**. The light mask is further described below in greater detail in conjunction with FIG. **1**A.

The system **10** also includes a camera **20** (or other type of detector) adapted to receive light represented by lines **22***a*, **22***b*, which lines are generally indicative of light beam boundaries. As further described below, the light **22***a*, **22***b* is associated with the transillumination light sources **12** and/or with the epi-illumination light source **16**. The camera **20** generates image information **28**, which is received by a normalization processor **30**. The normalization processor **30** generates normalized image information **32**, which is received by and displayed by a display **34**. The normalization processor **30** is further described below, where it will become apparent that the normalization provided by the normalization processor **30** results in improved "normalized" fluorescence images of a subject **24**.

In operation, the camera **16** receives the light **26***a*, **26***b*. In some embodiments, the transillumination light sources **18** and the epi-illumination light source **16** generate excitation light, **18**, **20**, respectively, concurrently and the camera **16** receives light **26***a*, **26***b* having contributions from both the transillumination light sources **12** and the epi-illumination light source **16** at the same wavelength as each of the excitation light sources **12**, **16**. In other embodiments, the transillumination light sources **12** and the epi-illumination light source **16** generate the excitation light **18**, **20**, respectively, at different times, so that at any particular time the camera **16** receives the light **26***a*, **26***b* from one of the transillumination light sources **12** and the epi-illumination light source **16**.

The light **26***a*, **26***b* received by the camera **16** in response to the excitation light **18**, **18***a*, **18***b* generated by the epi-illumination light source **16** can have contributions from the excitation light **18**, **18***a*, **18***b* at the excitation light wavelength and also contributions from emitted light (fluorescence or bioluminescence), emitted within the subject **24** at a different wavelength in response to the excitation light **18**, **18***a*, **18***b*. Contributions from the excitation light **18**, **18***a*, **18***b* can be used to form an intrinsic epi-illumination image, and contributions from the resulting emitted fluorescent light can be used to form a fluorescence epi-illumination image and also a normalized fluorescence epi-illumination image.

Similarly, the light **26***a*, **26***b* received by the camera **16** in response to the excitation light **14** generated by the transillumination light source **12** can have contributions from the excitation light **14** at the excitation light wavelength and contributions from emitted light (fluorescence or bioluminescence), emitted within the subject **24** at a different wavelength in response to the excitation light **14**. Contributions from the excitation light **14** can be used to form an intrinsic transillumination image, and contributions from the emitted light can be used to form a fluorescence transillumination image and also a normalized fluorescence transillumination.

From the above discussion, it should be recognized that the system **10** can generate normalized fluorescence epi-illumi-

nation images, normalized fluorescence transillumination images, or both normalized fluorescence epi-illumination images and normalized fluorescence transillumination images. The system **10** can also generate normalized bioluminescence images.

As described above, normalized fluorescence epi-illumination images can be generated by illuminating tissue (e.g. the mouse **24** in FIG. **1**) with the excitation light **18**, **18***a*, **18***b* generated by the epi-illumination light source **16**. The camera **20**, positioned on the same side of the tissue **24** as the epi-illumination light source **16**, is used to capture both excitation (epi-illumination) light reflected from the tissue **24** to form an intrinsic epi-illumination image, and fluorescent light, which is emitted from within the tissue **24** in response to the excitation (epi-illumination) light **18**, **18***a*, **18***b* to form an un-normalized fluorescence epi-illumination image. The intrinsic epi-illumination image and the un-normalized fluorescence epi-illumination image are combined by the normalization processor **30** to generate the normalized fluorescence epi-illumination image.

Also as described above, normalized fluorescence transillumination images can be generated by illuminating the tissue (here the mouse **24**) with the excitation light **14** generated by the transillumination light sources **12**. The camera **20**, positioned on the opposite side of the tissue **24** as the transillumination light sources **12**, is used to capture both excitation (transillumination) light passing through the tissue **24** to generate an intrinsic transillumination image, and fluorescent light, which is emitted from within the tissue **24** in response to the excitation (transillumination) light **14** to form an un-normalized fluorescence transillumination image. The intrinsic transillumination image and the un-normalized fluorescence transillumination image are combined by the normalization processor **30** to generate the normalized fluorescence transillumination image.

To generate normalized bioluminescence images, the system **10** collects an un-normalized image of bioluminescence light. In this case, bioluminescence light is spontaneously emitted and is not generated in response to the excitation lights **14**, **18**, **18***a*, **18***b*. The un-normalized bioluminescence image can be combined by the normalization processor **30** with either the intrinsic epi-illumination image or with the intrinsic transillumination image to generate the normalized bioluminescence image.

For either epi-illumination imaging or transillumination imaging, in order to form the intrinsic images in some embodiments, the excitation light source generates one or more wavelengths (i.e. incident light) including, but not limited to, a wavelength of excitation light, and the intrinsic image is generated by combining intrinsic images associated with the one or more wavelengths. In some arrangements the combination is a weighted average.

While the term "excitation light" is described above to be associated with light that can excite fluorescence in biological tissue, the excitation light can also be used by the system **10** in order to generate the normalized bioluminescence images. In some arrangements, the excitation light can be generated at the bioluminescence wavelengths so that it can be used to capture the propagation characteristics of the bioluminescence in the tissue. For example, as described above, in normalized bioluminescence transillumination imaging, the system **10** collects both an image of the excitation (transillumination) light (intrinsic image) and an un-normalized image of bioluminescence light.

As described above, normalized fluorescence transillumination imaging is provided by positioning the plurality of transillumination light sources **12** generally on the other side of the tissue **24** from the camera **20**. The transillumination light sources **12** can have an illumination pattern, for example, by using an array of transillumination light sources **12** disposed in a predetermined pattern, or by using an optical guiding system disposed in the predetermined pattern, for example, the light mask **26**

In fluorescence transillumination imaging using the plurality of transillumination light sources **12**, transillumination imaging can be accomplished by a superposition of light signals associated with excitation light generated by each one of the plurality of transillumination light sources **12**. In essence, each one of the transillumination light sources **12** can be sequentially illuminated and resulting intrinsic and fluorescence images can be sequentially captured.

The camera **20** can collect both excitation light associated with the light sources **12**, **16**, which is reflected from and/or which passed through the tissue **24**, and can collect emitted light associated with a fluorochrome within the tissue and/or associated with an endogenous tissue fluorescent molecule. The system **10** can also collect emitted bioluminescence light, for example, when the excitation light sources **12**, **16** are turned off. The same system **10**, using the same camera **20**, can be used to generate both normalized fluorescence epi-illumination images and normalized fluorescence transillumination images. The camera **20** is shown in greater detail in conjunction with FIGS. **2** and **2**A.

While it is described above that the epi-illumination light source **16** and the transillumination light sources **12** are adapted to generate excitation light, in other arrangements, the epi-illumination light source **16** and/or the transillumination light sources **12** are adapted to generate incident light having the wavelength of the excitation light and also other wavelengths.

Referring now to FIG. **1**A which is comprised of a first panel labeled "(a)" and a second panel labeled "(b)", a first exemplary light mask **26***a* shown in the panel (a) and a second exemplary light mask **26***b* shown in the panel (b). The masks **26***a*, **26***b* serve to block stray light and also excitation light that could otherwise directly hit the camera (or other detector) and saturate it or damage it. Additional absorbing materials can be used to selectively prevent excitation light **14**, **18** generated by the excitation light sources **12**, **16**, respectively, from directly entering the camera **20**. In other arrangements, selective beam-scan patterns can be used to avoid direct exposure of the camera **20** to excitation light **14**, **18**. In other arrangements, adaptive attenuation of light intensity from the plurality of transillumination light sources **12** can be used to prevent saturation of the camera **20** and also to improve the dynamic range of images acquired by the camera **20**. For example, dynamic range can be improved for light that is minimally attenuated by tissue, for example, light close to the tissue borders.

Referring now to FIG. **1**B, another system **50**, which is similar to the system **10** of FIG. **1**, but which includes a single transillumination light source **52** having a relatively wide beam of excitation light, represented by an arrow **54**, and by lines **54***a*, **54***b*, which are generally indicative of light beam boundaries. In other arrangements, the emitted light **54**, **54***a*, **54***b* could have a spatially attenuated pattern to prevent CCD pixel saturation close to and outside of borders of tissue **64**.

Other elements of FIG. **1**B can be the same as or similar to elements of FIG. **1**. For example, a mask **66** can be the same as or similar to the mask **26**, an epi-illumination light source **56** can be the same as or similar to the epi-illumination light source **16**, a camera **60** can be the same as or similar to the camera **20**, a normalization processor **70** can be the same as or

similar to the normalization processor **30**, and a display **74** can be the same as or similar to the display **34**.

For both the system **10** of FIG. **1** and the system **50** of FIG. **1**B, methods of generating "normalized" images are described below using "un-normalized" images. Generation of a normalized fluorescence epi-illumination images involves collection of at least two separate images (i.e., two types of image data) that are combined to provide the normalized fluorescence epi-illumination image (or normalized image data). The two images include an image of the tissue (e.g., **24**, FIG. **1**) resulting from epi-illumination excitation light (e.g., **18**, **18***a*, **18***b*, FIG. **1**) generated by an epi-illumination light source (e.g., **16**, FIG. **1**), which is generally reflected from the tissue **24** (FIG. **1**), and also an un-normalized image of emitted light from a fluorescent probe (i.e., a fluorochrome). The emitted light is excited by the excitation light **18**, **18***a*, **18***b*. The image of the excitation light **18**, **18***a*, **18***b* is also referred to herein as an intrinsic epi-illumination image and the image of the emitted light is also referred to herein as an un-normalized fluorescence epi-illumination image.

In some embodiments, two dark light images (i.e., background images that can contain, for example, stray light and noise) are also collected and used in combination with the above two images to generate the normalized fluorescence epi-illumination image. One dark light image can be generated using the same acquisition settings (gain, exposure time, etc.), which are used to capture the above-described intrinsic epi-illumination image, and the other dark light image can be generated using the same acquisition settings, which are used to capture the above-described un-normalized fluorescence epi-illumination image. Use of dark images will become apparent from equations below.

Similarly, generation of a normalized fluorescence transillumination image involves collection of at least two separate un-normalized images (i.e., two types of image data) that are combined to provide the normalized fluorescence transillumination image (or normalized image data). The two un-normalized images include an image of the tissue (e.g., tissue **24**, FIG. **1**) generated from transillumination excitation light (e.g., excitation light **14**, FIG. **1**) generated by a transillumination light source (e.g., source **12**, FIG. **1**), which passes through the tissue **24**, and an image of emitted light from a fluorescent probe (i.e., a fluorochrome). The emitted light is excited by the excitation light **14**. The image of the excitation **14** is also referred to herein as an intrinsic transillumination image and the image of the emitted light is also referred to herein as an un-normalized fluorescence transillumination image.

In some embodiments, two dark light images are also collected and used to generate the normalized fluorescence transillumination image. One dark light image can be generated using the same acquisition settings (gain, exposure time, etc.), which are used to capture the above-described intrinsic transillumination image, and the other dark light image can be generated using the same acquisition settings, which are used to capture the above-described un-normalized fluorescence transillumination image.

Similarly, for bioluminescence, at least two, but in some embodiments four, images can collected, i.e. the intrinsic image and dark image for excitation light (epi-illuminated or transilluminated) and the emitted image and dark image for bioluminescence.

For embodiments using dark images, the dark images need not be acquired in each measurement but can be acquired once and stored in memory. As described above, in some embodiments, the dark current images may not be used, especially if dark images have very low contrast. In some other embodiments, an equivalent subtraction method may be used.

For both normalized fluorescence epi-illumination imaging and normalized fluorescence transillumination imaging, the excitation light provided by the light source and the emitted light generated by the fluorescent probe can be at different wavelengths. The emitted light received to generate bioluminescence images and the excitation light employed in associated intrinsic light images can be at different wavelengths or at the same wavelength. In one particular embodiment, the emitted light is near infrared light and the excitation light is also near infrared light but at a shorter wavelength. In one particular embodiment of bioluminescence imaging, the emitted light contains visible and near-infrared spectral components and the excitation light contains similar spectral components.

The following equations can be used in a method to generate normalized and noise-reduced fluorescence epi-illumination images and normalized and noise-reduced transillumination fluorescence images. Such a method can be performed in a system similar to systems **10**, **50** described above in conjunction with FIGS. **1** and **1**B, respectively. It will be understood that, in some embodiments, the un-normalized images described below can be generated by the camera **20** of FIG. **1** or the camera **60** of FIG. **1**B. The normalized images described below (and also the noise reduced un-normalized images), which can be generated by further processing and/or combination of the un-normalized images, can be generated by the normalization processor **30** of FIG. **1** or the normalization processor **70** of FIG. **1**B.

An un-normalized and noise-reduced fluorescence epi-illumination image, Ie, can be expressed as:

$$I_e = I_{fe} - I_{fn} \quad \text{(Eq. 1)}$$

where I_{fe} is the fluorescence image generated when using an epi-illumination light source (e.g. light source **16**, FIG. **1**) and I_{fn} is the background camera noise image (i.e., a dark image or a constant), respectively. I_{fn} can be acquired with identical acquisition and experimental parameters as I_{fe} but in the absence of excitation light, or it can be approximated by an equivalent constant value, which can be based on an experimental measurement. Hereafter, the image, I_e, is referred to as an un-normalized noise-reduced fluorescence epi-illumination image, or more simply an un-normalized fluorescence epi-illumination image, where the noise is presumed.

Similarly an un-normalized and noise-reduced fluorescence transillumination image, I_t, can be expressed as:

$$I_t = \sum_{k=1}^{Ns} g(k)(I_{ft}(k) - I_{fn})_{>Tf} \quad \text{(Eq. 2)}$$

where Ns is the number of back-illuminating transillumination light sources (e.g., light sources **12**, FIG. **1**) where (Ns≥1), $I_{ft}(k)$ is a fluorescence transillumination image obtained by illuminating the k^{th} transillumination light source, and I_{fn} is a corresponding noise image (also including an offset) obtained under identical conditions but with no illumination by an excitation light source (i.e., a dark image). The factor g(k) is a percentage coefficient that is included to correct for the variation of individual transillumination light source strengths compared to a median source strength as calculated based on the relative strength of each source, which values are measured, for example, through a homogenous intralipid. Tf is a threshold value.

All fluorescence image values above the threshold, Tf, are summed together (superimposed) to yield the un-normalized noise-reduced fluorescence transillumination image. In one particular embodiment, the threshold, Tf, is set at 10 times the standard deviation of the photon counts seen in the I_{fn} image. The value of the threshold, Tf, is selected to prevent noise, or to prevent image signals having a low signal to noise ratio from being included in the un-normalized fluorescence transillumination image, I_t. Hereafter, the image, I_t, is referred to as an un-normalized noise-reduced fluorescence transillumination image, or more simply an un-normalized fluorescence transillumination image, where the noise subtraction may be presumed.

The above un-normalized fluorescence epi-illumination image, Ie, and the un-normalized fluorescence transillumination image, I_t, can be normalized in the following way. The normalization process divides the un-normalized images by corresponding intrinsic images (i.e. by an image of epi-illumination excitation light for epi-illumination imaging or by an image of transillumination excitation light for transillumination imaging).

A normalized fluorescence epi-illumination image, U_e, can be computed as:

$$U_e = \frac{a \cdot (I_{fe} - I_{fn})_{>Tf} + c_f}{b \cdot (I_{ee} - I_{en})_{>Te} + c_e} \quad \text{(Eq. 3)}$$

where I_{ee} is a epi-illumination image obtained at the excitation light wavelength (i.e., an intrinsic epi-illumination image, which is an image of the tissue generated from excitation light generated by a epi-illumination light source having reflected from the tissue) and I_{en} is camera noise and offset obtained with no excitation from the light source (or dark image).

Normalized image performance depends upon the threshold values Tf (for the fluorescence epi-illumination image) and Te (for the intrinsic epi-illumination image). In one particular embodiment, Ue is set to zero unless the I_{ee}–I_{en} denominator values are above the threshold, Te, and the I_{fe}–I_{fn} values are above the threshold, Tf. Selection of the thresholds can be based upon noise statistics or empirical data. The selection can be made statically by selecting values a predetermined number of noise standard deviations of the noise image (for example, 20 standard deviations of the noise image, I_{en}), or adaptively. The constants c_f and c_e can also be statically or adaptively selected, and their purpose is to stabilize the image. The constants c_f and c_e can have a value of zero, depending on user preferences, or they may be small values to provide small offset values to stabilize the ratio and yield a more accurate visual result. In one particular embodiment, a default setting of cf=0 and c_e=1 is used to avoid division by zero. Similarly, factors a and b can implement a-priori information on the relative strength of fluorescence and excitation images, for example, the relative attenuation of the filters used in fluorescence measurements, compared to the filters used in excitation light measurements (see FIG. **2** below). In another arrangement, a=1 and b=1. However, depending upon the application, a and b can be other values given by pre-determined equations (for example functions of wavelength) or they can be experimentally measured.

Similarly a corresponding normalized fluorescence transillumination image, U_t, can be computed as:

$$U_t = \frac{a \cdot \sum_{k=1}^{Ns} g(k)(I_{ft}(k) - I_{fn})_{>Tf} + c_f}{b \cdot \sum_{k=1}^{Ns} g(k)(I_{et}(k) - I_{en})_{>Te} + c_e} \quad \text{(Eq. 4)}$$

where I_{et} is a transillumination image obtained at the excitation wavelength (i.e., an intrinsic transillumination image, which is an image of the tissue generated from excitation light generated by a transillumination light source having passed through the tissue) and I_{en} is camera noise (dark image) obtained with no excitation from the excitation light source. Fluorescence and excitation transillumination images for each of the N_s sources employed are summed (superimposed) after subtraction with the noise images I_{fn} and I_{en} and application of the T_f and T_e thresholds, respectively.

While noises I_{fn} and I_{en} are subtracted in the above equations 3 and 4, it should be understood that the similar normalization techniques apply to a system in which one or both of the noises are not subtracted.

It should be noted that, for the system **50** described in conjunction with FIG. **1**B, which has only the one transillumination light source **52**, a single transillumination image is obtained. In this case Eq. 2 and Eq. 4 are written without the summation terms.

An un-normalized fluorescence transillumination image (one transillumination light source) can be expressed as:

$$I_t = (I_{ft} - I_{fn})_{>Tf} \quad \text{(Eq. 5)}$$

A normalized fluorescence transillumination image (one transillumination light source) can be expressed as:

$$U_t = \frac{a \cdot (I_{ft} - I_{fn})_{>Tf} + c_f}{b \cdot (I_{et} - I_{en})_{>Te} + c_e}, \quad \text{(Eq. 6)}$$

where I_{ft} and I_{et} are now single transillumination images.

The intrinsic image can be an image that is obtained at a broader wavelength, i.e. at wavelengths that not only include the wavelength of the excitation light (e.g., **14**, **18** of FIG. **1**) but also the wavelength of resulting fluorescent light. Alternatively two "intrinsic" images can be obtained, one at the wavelength of the excitation light and one at the wavelength of the emitted (fluorescence) light, wherein a combination of the two can be used for normalization, for example, a linear combination or a weighted product.

In the above-described case of intrinsic image generation in which the intrinsic image is obtained at the broader wavelength range that includes the fluorescence wavelength, the fluorescence image can first be subtracted from the intrinsic image obtained, i.e. an image generated using an excitation light having a spectral response that is similar to the excitation and emission spectral responses, i.e. I_{ee}=I_{ee}'–I_{fe} and I_{et}=I_{et}'–I_{ft}, where, as described above, I_{ee} is an epi-illumination image obtained at the wavelength range of the extended excitation light source, I_{et} is a transillumination image obtained at the wavelength range of the extended excitation light source, I_{fe} is the fluorescence image generated when using an epi-illumination light source, and, I_{ft} is the fluorescence image generated when using a transillumination light source. In this case, I_{ee}' and I_{et}' are intrinsic images over multiple wavelengths corresponding to the excitation and emission wavelengths obtained from epi-illumination and from transillumination, respectively, using excitation light having a wavelength at the excitation wavelength and also additional excitation light having a light spectrum that matches the wavelengths of emitted (fluorescence) light. The excitation light matching the fluorescence spectrum can be used to capture fluorescence propagation characteristics. This approach becomes particularly important for cases where the excitation light is in the visible wavelength range and the fluorescent light is in the near-infrared wavelength range. While the above is described

generically, significant accuracy can be achieved by appropriately selecting the range and response over which the I_{ee}' and I_{et}' are obtained.

For bioluminescence imaging, Eq. 1, Eq. 3, and Eq. 5 (un-normalized epi-illumination, normalized epi-illumination, and un-normalized fluorescence transillumination images, respectively) can remain substantially unchanged, but I_{fx} is interpreted to be associated with bioluminescence rather than with fluorescence. In normalized bioluminescence imaging, images can be normalized with intrinsic images obtained at a selected propagation wavelength range that best matches the wavelength of the bioluminescence systems. Therefore, excitation light can also include light having a spectrum that matches the wavelengths of the bioluminescence. Eq. 3 provides a normalized bioluminescence epi-illumination image.

For bioluminescence imaging, Eq. 2, used for multiple un-normalized fluorescence transillumination images, is not used. Also, for normalized transillumination bioluminescence imaging, Eq. 4, which applies to multiple transillumination light sources, can be instead expressed as:

$$U_t = \frac{a \cdot (I_{ft} - I_{fn})_{>Tf} + c_f}{b \cdot \sum_{k=1}^{Ns} g(k)(I_{et}(k) - I_{en})_{>Te} + c_e} \quad \text{(Eq. 7)}$$

and Eq. 6 for normalized bioluminescence transillumination imaging using one transillumination light source becomes:

$$U_t = \frac{a \cdot (I_{ft} - I_{fn})_{>Tf} + c_f}{b \cdot (I_{et} - I_{en})_{>Te} + c_e}. \quad \text{(Eq. 8)}$$

The modification in Eq. 7 (compared with Eq. 4) shows a non-dependence of the bioluminescence signal on the excitation light sources, therefore, the summation as a function of light sources is dropped. Similarly it should be pointed out that Eq. 7 and Eq. 8 are more generic in nature as they do not explicitly direct the geometry by which bioluminescence images are captured. However they assume that excitation light propagates from similar tissues as from which the bioluminescence signal is collected. One embodiment for normalized imaging given by Eq. 3 applied to bioluminescence of superficial structures, wherein bioluminescence images are corrected for tissue attenuation in epi-illumination mode.

It should be appreciated that the normalization methods described above are generic in nature and can be applied in both systems **10**, **50** of FIG. **1** and FIG. **1**B (and also systems shown in FIGS. **9**, **9**A, and **10**).

An alternative normalization approach referred to herein as a "per-source" normalization approach, which independently normalizes each source that is time, frequency, or wavelength encoded and then adds the normalized results together. This normalization can be implemented with any light source geometry, even if the light sources are placed on the sides or the top-side of the tissue being imaged.

In accordance with Eq. 4 and the above description, a per-source normalization image can be expressed as:

$$U_{psn} = \sum_{k=1}^{Ns} \frac{a \cdot (I_{ft}(k) - I_{fn})_{>Tf} + c_f}{b \cdot (I_{et}(k) - I_{en})_{>Te} + c_e} \quad \text{(Eq. 9)}$$

The thresholds T_f and T_e can be constant for all values k or can be $T_f = T_f(k)$ and $T_e = T_e(k)$, which indicates that each ratio, applied for each individual light source, is threshold differently. In one embodiment, T_e is an adaptive threshold that is scaled as a function of $I_{et}(k)$ intensity, for example $T_e = 0.1 * I_{et}(k)$ and T_f is a constant, for example, 5 times the standard deviation of noise. Image pixels that give denominator values that are less than T_e are not calculated into the summation.

For all equations 1-9, a normalized image may be further processed for display by logarithmically or exponentially relating a final normalized image to the above-calculated normalized image. A normalized image for display is selected depending on the dynamic range of the values in the above-calculated normalized image.

Subtraction of images in equations above can comprise subtracting pixel magnitudes of a dark image (noise image) from co-registered pixels of an un-normalized emitted light image (fluorescence or bioluminescence, epi-illumination or transillumination). Division of images in equations above can comprise dividing pixel magnitudes of an un-normalized emitted light image (fluorescence or bioluminescence, epi-illumination or transillumination) by magnitudes of co-registered pixels of an intrinsic excitation light image (epi-illumination or transillumination).

While it is described above that the epi-illumination light source **56** and the transillumination light source **52** are adapted to generate excitation light, in other arrangements, the epi-illumination light source **56** and/or the transillumination light source **52** are adapted to generate incident light having the wavelength of the excitation light and also other wavelengths. The same is true for other arrangements, for example, arrangements shown in FIGS. **9**, **10**, and **11**, which are described more fully below.

Referring now to FIG. **1**C, a normalization processor **80**, which may be the same as or similar to the normalization processors **30**, **70** of FIGS. **1** and **1**B, respectively, receives one or more intrinsic excitation light images on path **82** and one or more un-normalized emitted light images on path **83** and generates one or more of a variety of types of a normalized light images made available on signal paths **93-96**.

The intrinsic excitation light images on path **82** are received by an optional intrinsic image combining processor **84**. The intrinsic image combining processor **84** can be adapted to combine (e.g. superimpose) intrinsic transillumination images for systems having a plurality of transillumination light sources, e.g., the system **10** of FIG. **1**, and to generate a single intrinsic image at an output thereof on path **84***a*. In other arrangements, the intrinsic image combining processor **84** is adapted to combine intrinsic images generated at a plurality of wavelengths. For a system having only one transillumination light source, for example, the system **50** of FIG. **1**B, or a system for which the excitation light source generates light at only the excitation light wavelength, the intrinsic image combining processor **84** is not needed.

Similarly, the un-normalized emitted light images on path **83** are received by an optional emitted image combining processor **85**. The emitted image combining processor **85** is adapted to combine (e.g. superimpose) emitted transillumination images for systems having a plurality of transillumination light sources, e.g., the system **10** of FIG. **1**, and to generate a single un-normalized emitted light image at an output thereof on path **85***a*. For a system having only one transillumination light source, for example, the system **50** of FIG. **1**B, the emitted image combining processor **85** is not needed.

An intrinsic image noise reduction processor **86** is adapted to receive the single intrinsic image on path **84***a* and also a dark image on path **82***a* (background image). The intrinsic image noise reduction processor **86** is further adapted to

combine the single intrinsic image and the dark image to provide a noise-reduced intrinsic image on path **86***a*. In some embodiments in which noise reduction is not performed, the intrinsic image noise reduction processor **86** is not used and the single intrinsic image on path **84***a* is used in place of the noise-reduced intrinsic image on path **86***a*.

Similarly, an emitted image noise reduction processor **87** is adapted to receive the single un-normalized emitted light image on path **85***a* and also a dark image on path **83***a* (background image). The emitted image noise reduction processor **87** is further adapted to combine the single un-normalized emitted light image on path **85***a* and the dark image on path **83***a* to provide a noise-reduced un-normalized emitted light image on path **87***a*. In some embodiments in which noise reduction is not performed, the emitted image noise reduction processor **87** is not used and the un-normalized emitted light image on path **85***a* is used in place of the noise-reduced un-normalized emitted light image on path **87***a*.

When the noise-reduced intrinsic image on path **86***a* is a noise-reduced intrinsic epi-illumination image and when the noise-reduced un-normalized emitted light image on path **87***a* is a noise-reduced un-normalized fluorescence epi-illumination image, those images are provided to a fluorescence epi-illumination normalization processor **89** adapted to generate a normalized fluorescence epi-illumination image on path **93**.

When the noise-reduced intrinsic image on path **86***a* is a noise-reduced intrinsic transillumination image and when the noise-reduced un-normalized emitted light image on path **87***a* is a noise-reduced un-normalized fluorescence transillumination image, those images are provided to a fluorescence transillumination normalization processor **90** adapted to generate a normalized fluorescence transillumination image on path **94**.

When the noise-reduced intrinsic image on path **86***a* is a noise-reduced intrinsic transillumination image and when the noise-reduced un-normalized emitted light image on path **87***a* is a noise-reduced un-normalized bioluminescence transillumination image, those images are provided to a bioluminescence transillumination normalization processor **91** adapted to generate a normalized bioluminescence transillumination image on path **95**.

When the noise-reduced intrinsic image on path **86***a* is a noise-reduced intrinsic epi-illumination image and when the noise-reduced un-normalized emitted light image on path **87***a* is a noise-reduced un-normalized bioluminescence epi-illumination image, those images are provided to a bioluminescence epi-illumination normalization processor **92** adapted to generate a normalized bioluminescence epi-illumination image on path **96**.

The normalization processor **80** is adapted to generate the four types of images on paths **93-96**, wherein each one of the images can be noise reduced or not. Furthermore, the normalized fluorescence transillumination image provided on path **94** and the normalized bioluminescence transillumination image provided on path **95** can be generated using one excitation transillumination light source or a plurality of excitation transillumination light sources (superimposed via the processors **84**, **85**). However, in other arrangements, only one or more of the processors **89-92** are included in the normalization processor **80**.

Referring now to FIG. **2**, a camera **100**, which can be the same as or similar to the camera **20** of FIG. **1** and the camera **60** of FIG. **1**B, is adapted to receive and process light.

The light received by the camera **100** can include intrinsic excitation light resulting from excitation light generated by an epi-illumination light source, and/or fluorescent light emitted by a tissue in response to the excitation light generated by the epi-illumination light source. The light received by the camera **100** can also include excitation light resulting from excitation light generated by a transillumination light source, and/or fluorescent light emitted by a tissue in response to the transillumination light generated by the transillumination light source. The light received by the camera **100** can also include bioluminescence light naturally generated within the tissue.

The camera **100** can include an intrinsic image processor **102**, which is adapted to receive the above-described excitation light resulting from either the epi-illumination light source or the transillumination light source that has impinged upon and interacted with the tissue, and which is also adapted to generate an intrinsic image (i.e., intrinsic image data). The camera **100** can also include a fluorescence image processor **104**, which is adapted to receive the above-described fluorescent light resulting from either the epi-illumination light source or the transillumination light source that has been emitted from the tissue, and which is also adapted to generate an un-normalized fluorescence image (i.e., un-normalized fluorescence images data). The intrinsic image processor **102** and the fluorescence image processor **104** can be provided, for example, by way of or in conjunction with an optical filter (excitation light filter **106**) adapted to pass the excitation light and another optical filer (fluorescent light filter **108**) adapted to pass the fluorescent light. The intrinsic image processor **104** and the fluorescence image processor **106** can operate at substantially the same time or in sequence to provide the intrinsic image (i.e., intrinsic image data) and the un-normalized fluorescence image (i.e., un-normalized fluorescence image data). Since the excitation light received by the camera **100** may be at a greater intensity than the fluorescent light, in some embodiments, the excitation light filter **106** can include a light attenuating filter, for example, a neutral density filter.

In some embodiments, polarizers (not shown) can be further used to preferentially select polarization modes. The polarizers can be used at the epi-illumination light source (e.g. **56**, FIG. **1**B), at the transillumination light source (e.g., **52**, FIG. **1**B), at the intrinsic portion **102** of the camera **100**, and/or at the fluorescence processor **104** of the camera **100**. The polarizers can facilitate more superficial imaging (when the same orientation polarization filters are used at each end of a light path) or deeper imaging (when cross-polarizers are used). In addition, polarizers can be used to better separate emission (fluorescence or bioluminescence) from excitation light by polarizing the emission light with one polarization direction and detecting emitted light in with a cross polarization.

While the fluorescence image processor **104** is described above, it should be understood that the same processor can be used to receive light resulting from bioluminescence and to form a bioluminescence image. While the excitation light filter **106** is described above, in other arrangements, the excitation light filter **106** is replaced with an incident light filter adapted to pass a plurality of wavelengths.

Referring now to FIG. **2**A, another camera **150**, which can be the same as or similar to the camera **20** of FIG. **1** and the camera **60** of FIG. **1**B, is adapted to receive and process light. The camera **150** can include a filter wheel assembly **152** adapted to rotate and to select either an excitation light filter **154** or a fluorescent light filter **154**. An image processor **158** is adapted to generate an intrinsic image or an un-normalized fluorescence image accordingly.

Unlike the camera **100** of FIG. **2**, the camera **150** has only one image processor **158**, which is used to sequentially provide both the intrinsic image (i.e., intrinsic image data) and the un-normalized fluorescence image (i.e. un-normalized

fluorescence image data). Since the excitation light received by the camera **100** may be at a greater intensity than the fluorescent light, in some embodiments, the excitation light filter **154** can include a light attenuating filter, for example, a neutral density filter. While the excitation light filter **154** is described above, in other arrangements, the excitation light filter **154** is replaced with an incident light filter adapted to pass a plurality of wavelengths.

Before describing a series of images presented below, phantoms used to generate the images are described. A first phantom (used in images of FIGS. **3**, **3**A, and **4**) was relatively spatially-homogenous and included two diffusive and fluorescent tubes placed 5 mm apart, which were immersed in a chamber. The tubes were glass capillaries ~1.5 mm diameter sealed on one end, containing 1% intralipid solution, 25 ppm of India ink, and 200 nm of Cy 5.5 dye to simulate varying absorption and fluorescence emanating from a superficial tumor. The chamber was further filled with the same solution of intralipid and ink but without the fluorochrome. The first phantom was placed at various depths in the chamber and was used to assess the relative performance of the above described normalization method as a function of phantom depth.

A second phantom (used in images of FIGS. **5**-**5**C) employed the same relative homogenous background and a two-tube arrangement as the first phantom but employed two 3 mm diameter plastic tubes. The second phantom was used to examine accuracy of the two normalization methods as a function of varying optical properties. In these experiments the left tube was filled with 400 nm of Cy5.5 whereas the right tube contained 200 nm of Cy5.5. Both tubes and the background medium were filled with the same solution of 1% intralipid solution and 25 ppm of India ink (FIGS. **5** and **5**B). Subsequently the absorption of the left tube was increased to 2.5× the background absorption concentration (FIGS. **5**A and **5**C). The second phantom was used to examine robustness of the normalized imaging methods with respect to varying background optical properties.

A third phantom was used in a first animal experiment (shown in FIGS. **6** and **6**A) to generate images of a nude mouse post-mortem. This experiment demonstrated the merits of normalized transillumination in resolving deep-seated activity, better than epi-illumination. It also demonstrated how normalized epi-illumination can result in reducing false positives. The third phantom included a 1.8 mm diameter glass tube filled with intralipid and 400 nm of Cy5.5 dye, which was inserted in the animal through the esophagus until it reached the middle of the torso of the animal. Then the animal was placed in the imaging chamber and all image sets described by Eqs. 1-4 were obtained.

Images shown in FIG. **6**A and FIG. **7**A are negative images. However, positive images could equally well be generated and shown. It will also be understood that the intrinsic, un-normalized epi-illumination fluorescence, un-normalized transillumination fluorescence, normalized epi-illumination fluorescence, and normalized transillumination fluorescence images presented in FIGS. **6**, **6**A, **7**, and **7**A are all processed to remove background noise, for example, as in equations 1-4 above.

Referring now to FIG. **3**, normalized fluorescence epi-illumination images of the first phantom are shown in panels (a)-(e), where the above-described tubes are placed at different depths in the chamber as indicated at 0, 1, 3, 5 and 7 mm away from a front glass window of the chamber. At a depth of 0 mm the tubes are physically in contact with the front window. The images in panels (a)-(e) are scaled to their maximum, since signal intensity drops exponentially with depth.

In this particular experiment, fluorescence epi-illumination imaging quality appears reduced as a function of depth. The tubes of the first phantom are practically not detectable at a depth of 7 mm. However, normalized fluorescence epi-illumination imaging yields significant resolution of the tubes at 0 mm. The two tubes are clearly resolved at 0 mm (when they are in contact with the front window). In contrast, it will be shown below, that at 0 mm, the tubes are less well resolved in normalized fluorescence transillumination imaging.

Referring now to FIG. **3**A, normalized fluorescence transillumination images of the first phantom are shown in panels (a)-(e). Again, the images in panels (a)-(e) are scaled to their maximum since signal intensity drops exponentially with depth.

The tubes are detected in all normalized transillumination images at a variety of depths, although resolution appears to deteriorate as a function of depth. Broadening of the detected image of the tubes is seen in the fluorescence transillumination images as a function of depth as expected. Moreover, as the arrows indicate, there is a length dependent broadening as well. However the ability of normalized transillumination to detect the tubes deeper than normalized epi-illumination (FIG. **3**) is apparent and may be important for improving planar images of sub-surface activity and deep-seated activity.

Referring now to FIG. **4**, transillumination images of the first phantom are shown for the tubes placed at a depth of 5 mm. In panel (a), an intrinsic image of excitation light is generated. As described above, the excitation light corresponds to light from a transillumination light source having passed through tissue, here through the chamber and first phantom. The region of excitation light measures ~3×2 cm and its intensity exponentially drops outside this area.

In panel (b), an un-normalized fluorescence transillumination image is collected. As described above, the fluorescent light is emitted by a fluorescent probe within the tubes in response to the emitted light from the transillumination light source. The image of panel (b) is an un-normalized image, I_t, according to equation 2.

In panel (c), a normalized fluorescence transillumination image, U_t, is shown according to equation 4. The image, U_t, more accurately represents a true length of the tubes than the image, It, of panel (b). This is because the tubes are not covered homogeneously by the excitation light of panel (a). It would be advantageous to homogeneously illuminate the complete region of interest, however, as shown, the normalization can accurately represent dimensions of the tubes even when inhomogeneous excitation light is used. However, due to an asymmetry of the excitation light field of panel (a), there is an asymmetry of resolved "shape" at the ends of the image of the tubes (panel (c)).

Normalized fluorescence transillumination imaging is seen to be less sensitive to illumination field variations and also less sensitive to depth than normalized fluorescence epi-illumination imaging and than un-normalized fluorescence transillumination imaging. However, as shown by comparing FIG. **3**, panel (a), with FIG. **3**A, panel (a), normalized fluorescence epi-illumination imaging can be seen to provide higher resolution images than normalized fluorescence transillumination imaging for objects near a surface of a diffuse medium. This advantage is reduced even at a fluorescent probe depth of 1 mm below the surface.

Further experimental results described below show both normalized fluorescence transillumination images and normalized fluorescence epi-illumination images.

Referring now to FIGS. **5**-**5**C, images were obtained with fluorescence epi-illumination and fluorescence transillumi-

nation imaging of the second phantom described above. As described above, the second phantom includes two tubes immersed in a solution of 1% intralipid solution and 25 ppm of India ink. In FIGS. **5** and **5**B the tubes contained 25 ppm of India ink, i.e. the same as the background concentration. In FIGS. **5**A and **5**C the left tube contained 60 ppm India ink, i.e. ~2.5× the background ink concentration.

Referring first to FIGS. **5** and **5**A, panels show intrinsic (excitation) light images (panels (a)), un-normalized fluorescence epi-illumination images (panels (b)), and normalized fluorescence epi-illumination images (panels (c)), respectively. By comparing the un-normalized fluorescence epi-illumination images (panels (b)) with the normalized fluorescence epi-illumination images (panels (c)), the effect of added absorption of the left tube can be seen. At 2.5× concentration (FIG. **5**A) the un-normalized fluorescence image (panel b)) show only a 1.26:1 intensity ratio over the right tube where the actual ratio is 2:1 as shown at 1× concentration (panel b, FIG. **5**). However, the normalized fluorescence images (panels (c)) show an improved ratio of 1.41:1.

Referring now to FIGS. **5**B and **5**C, images are of the same phantom as in FIGS. **5** and **5**A including the above-described 1× or 2.5× ink concentration contained in the left tube as indicated. Panels show transillumination intrinsic images (panels (a)), un-normalized fluorescence transillumination images (panels (b)), and normalized fluorescence transillumination images (panels (c)), respectively.

Here, the un-normalized fluorescence transillumination images (panels (b)) show a 1.11:1 ratio, which is worse than the epi-illumination un-normalized fluorescence images (panels (b)) of FIGS. **5** and **5**A. However, the normalized fluorescence transillumination images (panels (c)) show a ratio of 1.58:1, which is the better than the normalized fluorescence epi-illumination images (panels (c)) of FIGS. **5** and **5**A. Therefore, normalized fluorescence transillumination imaging is shown to accurately measure fluorescence activity, here of superficial volumes, when the background optical properties are heterogeneous, as is the case when imaging most tumors and other diseases and medical conditions.

Referring now to FIG. **6**, panels (a)-(c) show an epi-illumination intrinsic image, an un-normalized fluorescence epi-illumination image, and a normalized fluorescence epi-illumination image, respectively, of a nude mouse implanted with a fluorescent tube post-mortem. Panel (a) also shows an approximate position of the tube (see dark arrows) in the viewing plane as indicated by a dashed outline (repeated in panels (b) and (c)), found by measuring the insertion distance and by post-experiment surgical removal. The normalized fluorescence epi-illumination image of panel (c) does not show the implanted tube. In fact, the normalized image of panel (c) has less contrast than the un-normalized image of panel (b).

A white arrow in panel (b) shows fluorescence where no fluorescence signal should be present in the un-normalized fluorescence image. This region of fluorescence is reduced in the normalized image of panel (c), as is also shown by a white arrow in panel (c). This reduction shows that the normalization method can reduce false positives.

Skin auto-fluorescence and bleed through signals (e.g., reception of direct light along paths from the light source to the camera) appear in standard un-normalized fluorescence epi-illumination images, and normalization provides a more uniform epi-illumination image with fewer artifacts. Therefore, a very low or no signal image in fluorescence is expected, since there is very low auto-fluorescence in the near infrared. This is shown by the low contrast normalized image (panel (c)).

Another advantage of the normalized fluorescence epi-illumination image is that it can better identify an absence of fluorescence. This is an important feature for intra-operative imaging, laparoscopic imaging, endoscopy, and small animal imaging, since it accounts for variations and reflections of the excitation light that may bleed through to the camera or that may selectively excite more background auto-fluorescence. Therefore normalized fluorescent epi-illumination imaging can offer improved imaging accuracy and performance when used for its negative predictive capability.

Referring now to FIG. **6**A, panels (a)-(c) show an intrinsic image, an un-normalized fluorescent transillumination image, and a normalized fluorescence transillumination image, respectively, of the animal of FIG. **6**. The tube (at arrows) is detected and it is better resolved in the normalized fluorescence transillumination image than in the normalized fluorescence epi-illumination image (panel (c), FIG. **6**). The tube size is overestimated in panel (c) but its two-dimensional location in the viewing plane is well resolved. Transillumination imaging has been performed with mice in the presence or absence of matching fluids with very similar results.

The images of FIGS. **6** and **6**A show that normalized transillumination imaging, and similarly normalized epi-illumination imaging, can operate in the absence of matching fluids. However, proper care should be taken to selectively attenuate light at the borders of the tissue and outside the borders of the tissue to yield optical matching to the dynamic range of the measurements to the light detector used.

Referring now to FIG. **7**, panels (a)-(c) show an intrinsic epi-illumination image, a un-normalized fluorescent epi-illumination image, and a normalized fluorescence epi-illumination image, respectively, of an animal bearing a surface tumor with an ellipsoid shape of 4 mm×3 mm long and short axis dimensions, respectively, as determined by caliper measurements. The tumor is an Erb2 positive spontaneous tumor in the left mammary fat pad (right side as shown on the image). The animal was injected with a cathepsin-sensitive fluorescent probe. The tumor is indicated by an arrow on the images. This tumor was highly vascular and highly absorbing and was therefore seen darker than surrounding tissue on the intrinsic epi-illumination image shown in panel (a). A corresponding un-normalized fluorescence epi-illumination image shown in panel (b) does not identify the tumor. The normalized fluorescence epi-illumination image shown in panel (c) provides an image that appears more flat (i.e., the same intensity throughout the animal) and the tumor is now identifiable. However the detection ability is reduced due to other fluorescence activity within the animal, i.e., outside of the tumor, resulting from fluorochrome outside of the tumor.

Referring now to FIG. **7**A, panels (a)-(c) show an intrinsic transillumination image, an un-normalized fluorescence transillumination image, and a normalized fluorescence transillumination image, respectively, of the animal of FIG. **7**. The normalized image of panel (c) shows a marked fluorescence increase associated with the tumor (arrow). The tumor is not visible in panel (b), the un-normalized fluorescence transillumination image.

The animal of FIGS. **7** and **7**A was injected with a fluorescence probe, therefore, it is expected that there is a remnant background fluorescence signal in tissues apart from the tumor. This higher background image signal is evident in panel (c) (compare to panel (c) of FIGS. **6**A and **6**B). However the detection improvement gained by the normalized fluorescence transillumination method is still evident.

The above system and methods can also apply to bioluminescence imaging, wherein an un-normalized bioluminescence image is normalized with an intrinsic epi-illumination

image to yield a normalized bioluminescence epi-illumination image. While tissue emission apart from a tumor is less of an issue for bioluminescence than for fluorescence (autofluorescence), the normalization can nevertheless correct for attenuation heterogeneity across the animal surface, which can result from blood concentration heterogeneity, especially if a light source with spectral characteristics to match those of the emitted bioluminescence is used. Similarly the bioluminescence image can be normalized with an intrinsic transillumination image, and this may achieve a better result that than normalizing with an intrinsic epi-illumination image, especially when the bioluminescence is emitted from lesions deeper in the animal.

Referring now to FIG. **8**, images show another mouse having an Erb2 positive spontaneous tumor, which was imaged after the administration of a cathepsin sensitive activatable fluorescence probe. These images demonstrate improved images obtained with normalized epi-illumination imaging, which allow improved identification of the location of a fluorescent lesion (at arrows). It can be seen that normalized epi-illumination imaging can be useful for lesion identification when used in surgical operations, endoscopy, and laparoscopy. Here normalized images both before (panel (c)) and after (panel (d)) noise removal are shown.

Referring now to FIG. **8**A, images show a mouse having two HT1080 tumors implanted in the left and right side of the mammary fat pads, which are imaged after the administration of a cathepsin sensitive activatable fluorescence probe. These images demonstrate improved images obtained with normalized epi-illumination imaging, which allow improved identification of tumor borders. It can be seen that normalized epi-illumination imaging can be useful for border identification when used in surgical operations, endoscopy, and laparoscopy. Images both before (panel (c)) and after (panel (d)) noise removal are shown.

FIGS. **8** and **8**A show images related to techniques, which can be used in medical procedures, for example, intra-operative imaging, endoscopy, or laparoscopy used for the identification of lesions and their borders.

Referring again to FIG. **8**, panels (a)-(d) show an epi-illumination intrinsic image, an un-normalized epi-illumination fluorescent light image, a normalized fluorescence epi-illumination image that assumes noise thresholds at zero, and a normalized fluorescence epi-illumination image that has been processed with noise threshold operations as described in Eq. 3, respectively. The images show an animal bearing a surface tumor, as indicated with an arrow, which appears dark on the intrinsic epi-illumination image (panel (a)). Due to tumor absorption of light, the tumor appears dark on the un-normalized fluorescence epi-illumination image (panel (b)). Some activity that can be observed at the top part of the tumor is not co-localized with the presence of the tumor, even if the animal has been injected with the same activatable fluorescent probe as in FIG. **7**. Such activity at the borders of the tumor is typical in some un-normalized images and may be due to a local excitation field variation resulting from tissue heterogeneous absorption. Conversely the normalized fluorescence epi-illumination images of panels (c) and (d) correctly identify the location and size of the tumor. The image of panel (d) has been enhanced by the application of noise thresholds and by maximizing contrast. An additional smaller lesion also appears on the left side of the large tumor and this is visualized well in both normalized fluorescence images. However, in particular, the image of panel (d) demonstrates how the normalized fluorescence image can better show the total background fluorescence activity.

Referring again to FIG. **8**A, images show the same configuration as shown in FIG. **8**. A mouse has two HT1080 implanted tumors, which are indicated by arrows. Borders of the right tumor are not well differentiated since light "leaks" into the low absorbing skin fold that forms just above it. Therefore, the tumor appears as elongated. This characteristic may be drawback in surgical procedures where the exact tumor margins need to be identified so that unnecessary tissue damage during operation is minimized, especially, for example, in brain surgery. Conversely the normalized images of panels (c) and (d) better differentiate the tumor margins, which was also was correlated by actual tumor excision and invasive inspection.

Referring now to FIG. **9** a system **200** for intra-operative (i.e., imaging during an operation) procedures includes a camera **202**, which can include two lenses and/or filters **204***a*, **204***b*. The camera **202** with filters **204***a*, **204***b* can be the same as or similar to the camera **100** (including filters **106**, **108**) of FIG. **2**. The system **200** can also include an epi-illumination light source **206**, which can, in one particular embodiment, be in the form of a ring illuminator. The camera can provide un-normalized images to a normalization processor **208**, which can provide normalized images to a monitor **210**.

It will be appreciated that the system **200** is similar to the system **10** of FIG. **1**, however, the system **200** can be reduced in size so as to provide a system that can be more easily used during an operation.

In use, a body part or organ **212** can be exposed as shown, for example, by a surgical incision. A fluorochrome, which can be injected into the patient before or during the operation, and which tends to congregate at the site of a lesion **216**, makes the lesion fluorescently reactive. As described above, the camera **202** can generate an intrinsic epi-illumination image and an un-normalized fluorescence epi-illumination image. The normalization processor **208** can generate a normalized epi-illumination fluorescence image and can send the normalized image to an intra-operative monitor **210**, where it can be viewed by a surgeon.

In one particular embodiment, the intra-operative monitor **210** is in proximity to the operation theater so that the surgeon can see fluorescence indicative of disease on the monitor.

Also shown in FIG. **9**, an illuminator/laparoscope/endoscope **218** (hereafter called a probe **218**) can be used in a variety of ways. In one arrangement, the probe **218** provides a transillumination light source, which can be used so that the camera **202** can capture an intrinsic transillumination image and/or an un-normalized fluorescence transillumination image of a lesion **220**. This probe can be used during surgery to complement the epi-illumination system **200** or it can provide only transillumination imaging without use of the epi-illumination light source.

In another arrangement, the probe **218** provides an epi-illumination light source, wherein the system **200** is coupled to a distal end of the probe **218** in order to generate both an intrinsic epi-illumination image and an un-normalized fluorescence epi-illumination image and also a normalized fluorescence epi-illumination image of the lesion **220** in response to the epi-illumination light source provided by the probe **218**. Such arrangements will be better understood form the discussion below in conjunction with FIGS. **11** and **11**A.

In some arrangements, the probe **218** can be manually manipulated during the operation to image selected structures or organs. Real-time normalization techniques, such as the one described by Eq. 4, can be implemented using fast image processing digital processors, and can provide accurate imaging independent of the exact pattern of the illumination field,

which may vary when generating different illumination orientations when manipulating the probe **218**.

The probe **218** can be placed, for example, on the side of or behind a lymph node to investigate or confirm the presence or absence of a fluorochrome indicative of a malignancy. The probe **218** can be placed behind an organ to identify fluorescence activity indicative of function or disease. This fluorescence is provided by a local or systemic administration of a fluorochrome.

In some embodiments, as described above, the probe **218** is an endoscope or laparoscope so that the fluorescence activity can be captured by the probe **218**. These arrangements can be used, for example, in laparoscopy, minimally invasive surgery, endoscopy, and colonoscopy.

Referring now to FIG. **9**A, in another embodiment, the system **200** of FIG. **9** is constructed entirely or in part within a miniaturized detection and visualization system, for example, goggles **230** or a transparent screen (not shown). These embodiments can allow, via the use of appropriate filters, co-registration of anatomical contrast, as would be naturally seen by the eye, in combination with fluorescence activity. For example, in one arrangement, the body/organ **212** is seen naturally and color contrast is added to indicate the presence of the fluorescence associated with a lesion.

In yet another embodiment, the system **200** is a portable system, for example a hand-held scanner.

Referring now to FIG. **10**, a system **250** includes a transillumination light source **252** disposed on the opposite side of a tooth **254** from a camera **256**. The camera **256** can have two filters **258***a*, **258***b*, and can be the same as or similar to the camera **100** of FIG. **2**. The camera **256** can generate an intrinsic transillumination image and also and un-normalized fluorescence transillumination image of the tooth **254** and structures therein. In some arrangements the camera is instead an endoscope, which can be the same as or similar to the probe of FIG. **9**. A normalization processor (not shown) and monitor (not shown) can be coupled to the camera **256** in order to generate normalized images.

In this embodiment, the transillumination light source **252** and the camera **256** are introduced in the mouth and disposed about the tooth **254** as shown. In some arrangements, the camera **256** also includes an epi-illumination light source (not shown), which can be the same as or similar to the epi-illumination light source **206** of FIG. **9**.

The system **250** can generate normalized fluorescence transillumination images and/or normalized fluorescence epi-illumination images of dental fluorescence contrast. In some embodiments, the system **250** can use native tissue auto-fluorescence, which can be excited by the excitation light source **252**. However, in other embodiments, extrinsically or topically administered external fluorochromes with molecular or functional specificity can be applied to the tooth **254**.

The system **250** can also be used to investigate other oral cavity diseases, for example gum disease or oral cancers. The system **250** can be used in combination with other non-invasive imaging systems, for example X-ray systems, to provide further diagnostic capability.

Referring now to FIG. **11**, a system **270**, used for colposcopic procedures includes a colposcopic probe **272** (or more simply a probe **272**) having a face **272***a*. The face **272***a* of the probe **272** is described in greater detail below in conjunction with FIG. **11**A. The probe **272** is inserted into the colon **274** of a patient and is coupled with a colposcope **276** to a colposcope coupler **278**. The colposcope **276** includes at least one light fiber (not shown) therewithin.

The colposcope coupler **276** is optically coupled to a light coupler **280**, which is adapted to receive light from at least one excitation light source (not shown) and to deliver the light to the colposcope **276**. Here, two illumination lights are shown, which are represented by arrows **282**, **284**. In some embodiments, illumination channel **1 282** provides epi-illumination excitation light at an excitation light wavelength, which is associated with normalization system and method of the present invention. In some embodiments, illumination channel **2 284** also provides epi-illumination excitation light at the same excitation light wavelength. However, in some embodiments, the illumination channel **2 284**, can provide light at another wavelength, for example, visible light wavelengths, and is associated with other optical functions, for example, direct colposcopic viewing of visible light as will be apparent from discussion below.

In further embodiments, the epi-illumination channel **2** provides another excitation epi-illumination light at another excitation light wavelength. In these embodiments, the first illumination channel **1 282** is used to excite certain fluorochromes within the colon **274** and the second illumination channel **284** is used to excite other fluorochromes within the colon **274**. With this arrangement, two different normalized fluorescence images can be generated.

The colposcope coupler **276** is also optically coupled to a light splitter **288**, which is adapted to pass a first portion **288** of received light to a camera/monitor **290**. The camera/monitor **280** can be used, for example, for direct colposcopic viewing of visible light during a procedure. A second portion **291** of the received passes through a light filter **292**, which is coupled to an optical coupler **294**, which directs the second received light **291** to a camera **298**. The camera **298** can be the same as or similar to the camera **100** of FIG. **2** or the camera **150** of FIG. **2**A. From the above discussion, it should be appreciated that the first and second portions **288**, **291**, respectively, of the received light can include excitation epi-illumination light at an excitation light wavelength, fluorescence light a fluorescence wavelength, epi-illumination light at another excitation light wavelength, fluorescent light at another fluorescence wavelength, and visible light, in any combination.

The camera **298** is shown to be most similar to the camera **100** of FIG. **1**, and has an intrinsic image processor **300** and a fluorescence image processor **302**. With this arrangement, the filter **292** can be the same as or similar to the excitation light filter **106** and the fluorescent light filter **108** of FIG. **2**.

The camera provides intrinsic excitation epi-illumination images **304** and un-normalized fluorescence epi-illumination images **304** to a normalization processor **306**. The normalization processor **306** can be the same as or similar to the normalization processors **30**, **70**, **80** of FIGS. **1**, **1**B, and **1**C, respectively. The normalization processor **306** is adapted to generate normalized fluorescence epi-illumination images **308** to a monitor **310**.

The system **270** is but one example of a system that can be used intra-operatively to provide normalized fluorescence epi-illumination images. Other similar systems can be used to provide normalized fluorescence transillumination images, as described above in conjunction with FIG. **9**.

A system similar to the system **270** can be used in a variety of operative procedures, including, but not limited to, a laparoscopic procedure (wherein the probe **272** is a laparoscopic probe), an endoscopic procedure (wherein the probe **272** is an endoscopic probe), a colonoscopic procedure (wherein the probe **272** is a colonoscopic probe), a colposcopic procedure (wherein the probe **272** is a colposcopic probe), an esophageal procedure (wherein the probe **272** is an esophageal

probe), a pulmonary procedure (wherein the probe **272** is a pulmonary probe), an oral procedure (wherein the probe **272** is an oral probe), and a dental procedure (wherein the probe **272** is a dental probe). A system similar to the system **270** can be used to image any internal body cavity within a patient.

Referring now to FIG. **11**A, the face **272***a* of the probe **272** (FIG. **11**) can include a first illumination port **350** from which the illumination light **282** emanates, and a second illumination port **352** from which the illumination light **282** emanates. The face **272***a* can also include a port having a camera lens **354**, which provides the portions **288**, **291** (FIG. **11**) of received light to the camera **298** (FIG. **1**). The face **272***a* can also include an irrigation port **356** through which fluid can be pumped into the colon **274** (FIG. **1**) or retrieved from the colon **274**. The face3 **272***a* can also include a channel **358** through which instruments can be inserted into the colon **274**.

The term "optical biopsy" used in conjunction with reference designator **358** is used to indicate an optical method, which is used to non-invasively acquire tissue information similar to the way in which information is obtained by a traditional invasive biopsy (i.e. tissue excision and histological examination). For example, an optical biopsy can be a spectroscopic technique that locally assesses cell nuclear size and density, an in-vivo confocal microscopy technique, or a normalized fluorescence method such as the one described herein. Similar to an invasive biopsy, an optical biopsy is used to characterize a relatively small lesion. A biopsy is used to characterize a relatively small area with high specificity in contrast to so-called "screening" that is associated with large fields of view.

It should be appreciated that all of the ports, and channels of the face **272***a* are associated with light fibers or channels through the colposcope **276** of FIG. **1**.

It will be appreciated from the image examples provided in FIG. **5**-**8**A, that normalized epi-illumination and normalized fluorescence transillumination imagining provide improved images in a variety of applications. Normalized epi-illumination imaging tends to be surface weighted. In other words, surface features can be seen well and with epi-illumination imaging the technique is appropriate for surgical and endoscopic applications. However the sensitivity and imaging resolution of epi-illumination imaging can be reduced for objects that are under the surface, in which case, normalized fluorescence transillumination imaging may be preferred. Normalized fluorescence transillumination imagining can often provide useful images in diffusive volumes to greater depths than normalized fluorescence epi-illumination imaging. This is because normalized fluorescence transillumination imagining has different characteristics than normalized fluorescence epi-illumination imaging. In particular, only a relatively small amount of excitation light is collected in normalized fluorescence transillumination imagining due to attenuation of the excitation light as it passes through the diffusive volume, therefore, any bleed through signal is reduced. Similarly, in normalized fluorescence transillumination imagining, any surface auto-fluorescence is excited by significantly attenuated light compared to normalized fluorescence epi-illumination imaging. These differences yield more uniform volume sampling in transillumination and significantly reduced background signals.

The above-described normalization can improve imaging performance by correcting for the effects of optical property variation in tissues and for variations in the strength of the excitation illumination field. Normalization is well suited for in-vivo investigations and can be implemented as a real time correction so that the user can observe corrected images in real time.

In general, it will be appreciated that un-normalized transillumination images can serve as raw data for conventional tomographic images. When used for improved tomographic imaging, normalized fluorescence transillumination images can provide improved image volume (i.e. increased depth), minimization of surface fluorescence, and reduced sensitivity to variations of transillumination light source optical properties. Due to the use of accurate photon propagation models and subsequent inversion, tomography using normalized images in place of un-normalized images can yield improved three-dimensional quantified maps of fluorescence activity and can correct for depth dependent sensitivities of collected photons. Therefore, tomography is a more integrated approach than either epi-illumination or transillumination planar imaging. Tomography is, however, more complex to implement and uses relatively complex tomographic processing. Tomography also suffers from longer computation times, which tend to make tomography less favored when compared to epi-illumination and transillumination planar approaches, especially when used for real time imaging.

It should be also appreciated that imaging performance can be significantly improved if normalized fluorescence epi-illumination and normalized fluorescence transillumination images and methods are used in combination. Using a combined normalized epi-illumination/normalized transillumination system as shown, for example, in FIGS. **1** and **1**B, the sensitivity of epi-illumination imaging to surface activity is retained, but volume sampling (i.e., resolution with depth) capability of transillumination imaging is added.

In both epi-illumination and transillumination systems and methods, normalization can offer certain advantages, including, but not limited to, reduced sensitivity to inhomogeneous illumination field variations and variations in background optical properties. Hardware requirements are only moderately increased for a combined epi-illumination and transillumination system.

All references cited herein are hereby incorporated herein by reference in their entirety.

Having described preferred embodiments of the invention, it will now become apparent to one of ordinary skill in the art that other embodiments incorporating their concepts may be used. It is felt therefore that these embodiments should not be limited to disclosed embodiments, but rather should be limited only by the spirit and scope of the appended claims.

What is claimed is:

1. A method of imaging, comprising:

generating incident light including excitation light with an excitation light source, wherein the excitation light source comprises an epi-illumination light source;

directing the incident light toward a tissue;

receiving the incident light with a light detector after the incident light has interacted with a tissue;

receiving emitted light with the light detector, wherein the emitted light is emitted from the tissue;

generating an excitation image of the tissue in response to received incident light, wherein the excitation image is obtained at a wavelength of the excitation light, wherein the excitation light is near-infrared light;

generating an un-normalized emitted light image of the tissue in response to the received emitted light, wherein the un-normalized emitted light image comprises an un-normalized fluorescence epi-illumination image generated at a selected wavelength different than the wavelength of the excitation light; and

dividing the un-normalized emitted light image by the excitation image to generate a normalized emitted light image of the tissue.

2. The method of claim 1, further comprising: inserting a probe into a patient during an operative procedure, wherein the generating the incident light includes transmitting the incident light from the probe, wherein the receiving the incident light includes receiving the incident light with the probe, and wherein the receiving the emitted light includes receiving the emitted light with the probe.

3. The method of claim 2, wherein the operative procedure comprises imaging an internal body cavity of a patient.

4. The method of claim 1, wherein the method comprises a selected one of a laparoscopic imaging method, an endoscopic imaging method, a colonoscopic imaging method, a colposcopic imaging method, an esophageal imaging, method, a pulmonary imaging method, an oral imaging method, and a dental imaging method.

5. The method of claim 1, wherein:

the emitted light comprises fluorescent light associated with the tissue;

the excitation image comprises an excitation epi-illumination image; and

the normalized emitted light image comprises a normalized fluorescence epi-illumination image.

6. The method of claim 5, wherein fluorescent light is generated by at least one of a fluorescent marker which is administered to the tissue and an endogenous tissue fluorescent molecule in response to the excitation light.

7. The method of claim 6, wherein the incident light is substantially at one wavelength.

8. The method of claim 1, wherein the dividing comprises dividing pixel magnitudes of pixels of the un-normalized emitted light image by pixel magnitudes of co-registered pixels of the excitation image.

9. The method of claim 1, wherein the incident light has a wavelength in the range of four hundred to one thousand nanometers and the emitted light also has a wavelength in the range of four hundred to one thousand nanometers, wherein the wavelength of the excitation light is shorter than the wavelength of the emitted light.

10. The method of claim 1, wherein the light detector comprises a charge coupled device camera.

11. The method of claim 1, further including:

generating a first background image of the tissue at generally a same wavelength as the excitation image;

generating a second background image of the tissue at generally a same wavelength as the un-normalized emitted light image;

combining the excitation image and the first background image to provide a noise-reduced excitation image;

combining the un-normalized emitted light image and the second background image to provide a noise-reduced un-normalized emitted light image; and

combining the noise-reduced un-normalized emitted light image and the noise-reduced excitation image to provide a noise-reduced normalized emitted light image.

12. The method of claim 1, wherein the incident light includes one or more wavelengths, and wherein the excitation image is generated by combining excitation images associated with the one or more wavelengths.

13. The method of claim 1, wherein the light detector is disposed on generally a same side of the tissue as the epi-illumination light source.

14. A system for imaging a tissue, the system comprising:

an excitation light source adapted to generate incident light including excitation light, wherein the excitation light source comprises an epi-illumination light source;

a light detector adapted to receive the incident light after the incident light has interacted with the tissue, the light detector further adapted to receive emitted light, wherein the emitted light is emitted from the tissue; and

one or more processors adapted to:

(i) generate an excitation image of the tissue in response to received incident light, wherein the excitation image is obtained at a wavelength of the excitation light, wherein the excitation light is near-infrared light;

(ii) generate an un-normalized emitted light image of the tissue in response to the received emitted light, wherein the un-normalized emitted light image comprises an un-normalized fluorescence epi-illumination image generated at a selected wavelength different than the wavelength of the excitation light; and

divide the un-normalized emitted light image by the excitation image to generate a normalized emitted light image associated with the tissue.

15. The system of claim 14, further comprising:

a probe; and

at least one optical fiber coupled between the probe and the excitation light source and between the probe and the light detector, wherein the at least one optical fiber is adapted to carry the incident light to the probe, to receive the incident light with the probe after the incident light has interacted with the tissue, and to receive the emitted light with the probe.

16. The system of claim 14, further comprising:

a probe configured to image an internal body cavity of a patient.

17. The system of claim 16, wherein the probe comprises a selected one of a laparoscopic probe, an endoscopic probe, a colonoscopic probe, a colposcopic probe, an esophageal probe, a pulmonary probe, an oral probe, and a dental probe.

18. The system of claim 14, wherein:

the emitted light comprises fluorescent light associated with the tissue;

the excitation image comprises an excitation epi-illumination image; and

the one or more processors are adapted to generate the normalized emitted light image as a normalized fluorescence epi-illumination image.

19. The system of claim 18, wherein fluorescent light is generated by at least one of a fluorescent marker which is administered to the tissue and an endogenous tissue fluorescent molecule in response to the excitation light.

20. The system of claim 19, wherein the incident light is substantially at one wavelength.

21. The method of claim 14, wherein the one or more processors are adapted to combine the un-normalized emitted light image and the excitation image by dividing pixel magnitudes of pixels of the un-normalized emitted light image by pixel magnitudes of co-registered pixels of the excitation image.

22. The system of claim 14, wherein the incident light has a wavelength in the range of four hundred to one thousand nanometers and the emitted light also has a wavelength in the range of four hundred to one thousand nanometers, wherein the wavelength of the excitation light is shorter than the wavelength of the emitted light.

23. The system of claim 14, wherein the light detector comprises a charge coupled device camera.

24. The system of claim 14, further including:

a first optical filter coupled to the light detector and adapted to pass the excitation light, wherein the excitation light has a first wavelength; and

a second optical filter coupled to the light detector and adapted to pass the emitted light, wherein the emitted light has a second wavelength different from the first wavelength.

25. The system of claim **14**, further including an optical filter coupled to the light detector and adapted to pass the excitation light and the emitted light.

26. The system of claim **14**, wherein the light detector is further adapted to generate a first background image of the tissue at generally a same wavelength as the excitation image and adapted to generate a second background image of the tissue at generally a same wavelength as the un-normalized emitted light image, and wherein the one or more processors are adapted to:

(i) combine the excitation image and the first background image to provide a noise-reduced excitation image;

(ii) combine the un-normalized emitted light image and the second background image to provide a noise-reduced un-normalized emitted light image; and

(iii) combine the noise-reduced un-normalized emitted light image and the noise-reduced excitation image to provide a noise-reduced normalized emitted light image.

27. The system of claim **14**, wherein the incident light includes one or more wavelengths, and wherein the excitation image is generated by combining excitation images associated with the one or more wavelengths.

28. The system of claim **14**, wherein the light detector is disposed on generally a same side of the tissue as the epi-illumination light source.

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UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 9,226,645 B2
APPLICATION NO. : 12/973075
DATED : January 5, 2016
INVENTOR(S) : Vasilis Ntziachristos

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the Claims

Column 29, Line 13, Claim 4, delete "imaging," and insert -- imaging --

Column 30, Line 50, Claim 21, delete "The Method of claim" and insert -- The system of claim --

Signed and Sealed this
Thirty-first Day of January, 2017

Michelle K. Lee

Michelle K. Lee
Director of the United States Patent and Trademark Office